Cryptococcal Meningitis in Viet Nam

Thesis

UNRESTRICTED

CRYPTOCOCCAL MENINGITIS

IN

VIET NAM

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Abstract

Cryptococcal neoformans is the commonest cause of invasive fungal disease in the world, causing up to 1.5 million cases of meningitis each year, and 1 million deaths. The aims of this thesis were to understand the differences in cryptococcal meningitis in Viet Nam in relation to the HIV infection status of the host, focussing on disease clinical phenotype, prognostic factors and the molecular epidemiology of the infecting isolates. In addition, the thesis examines the antifungal susceptibilities of Vietnamese *Cryptococcus* spp, how these vary by species and time, and the clinical utility of their measurement.

Patients drawn from two prospective cohorts (HIV infected and HIV uninfected) form the basis of the thesis. The HIV uninfected patients are predominantly immunocompetent. Clinical and investigational findings are compared in relation to the literature. HIV patients had shorter durations of history and more frequent headache, but there were few differences in clinical presentation. Previously published prognostic factors were reassessed; only 3 of 40 (altered consciousness, cerebrospinal fluid (CSF) white cell count, and CSF cryptococcal antigen titre) were independently associated with outcome.

Mortality was significantly higher in HIV infected patients, but the shapes of the survival curves were similar for both groups, most deaths occurring in the first 4 weeks of treatment.

Amplified Fragment Length Polymorphism analysis was used to define the relationships between HIV positive and negative strains – a clade of *C. neoformans* var *grubii* with increased propensity to cause disease in immunocompetent patients was identified.

The Sensititre® YeastOne® system was used to determine MICs of 7 antifungal drugs. The susceptibility of *C. neoformans* has decreased significantly over a 13 year period. No relationship with the rate of clearance of yeast from CSF and antifungal MIC was found, but raised amphotericin B MIC after 48 hours incubation may be associated with early death and warrants further investigation.

Acknowledgements

The work in this thesis would not have been possible without the participation of the adult patients recruited to the clinical studies on the Viet-Anh ward and ward D. I am indebted to them for their consent to enter the studies described in the thesis.

Many people at the Hospital for Tropical Diseases have helped care for the patients described. Dr TTH Chau co-ordinated the clinical care of the patients on the Viet-Anh ward and ensured the study notes were kept accurate and up to date. She was responsible for the collection of the clinical data of the HIV uninfected patients described in the thesis. Drs Dung and Mai co-ordinated the care of the HIV patients after transfer to ward D. I am extremely grateful to all the doctors, nurses and diagnostic staff (particularly PT Diep) on both wards – without them this work would not have been possible.

I am particularly grateful to Professor David Lalloo for enthusiasm, ideas, advice and a momentous introduction to Professor Jeremy Farrar, without whom none of this would have been possible. I had not expected a visit to OUCRU to discuss *Cryptococcus* strains to result in the opportunity to live and work in Viet Nam – a dream job for a young registrar in infectious diseases – and I will always be thankful for this fantastic opportunity, a great training in clinical research, his enthusiasm, ideas and encouragement, and his appointment of the unit manager. I would like to thank Professor TT Hien, for advice, patience and humour. Thanks especially to Jim Campbell for patient instruction in the art of classical mycology and covering the lab when I was away. Dr Marcel Wolbers, our statistician, was a huge resource and I thank him for patiently reminding me of the maths I had forgotten and instruction in statistical methods

and the use of R. As well as advice on the analyses in Chapters 3 and 5, he developed the non-linear effects model to describe the rates of decline in yeast counts in CSF described in Chapter 5. I am grateful to Associate Professor Dee Carter of the University of Sydney for help, advice, hosting me in her lab to learn about AFLP, and opening her home to me. Thanks also to Tien Bui, her research assistant, and to Wieland Meyer for providing control strains. Thanks to Steve Baker, for advice on all things molecular, enthusiasm and encouragement. Thanks to Duong Van Anh and Hoang Nha Thu, who have proved such willing and able mycology technicians, and thanks to Jeanne Packer in Oxford who always rapidly responded to requests for past publications. Thanks to Ruth Chambers for help with formatting.

Finally, thanks to my wife Mary, and children Megan and Tilly, for patience, support, coping with lone parenthood as this thesis has taken shape, and other things too many to mention.

Declaration

During this research I established the Research Mycology Laboratory at Oxford University Clinical Research Unit which I manage. The classical and molecular techniques described in this thesis were carried out in this lab. I established and problem-solved all the research tools within the laboratory. Since the end of 2007 the lab has employed 2 technicians that I have trained in these methods, and who now continue the sensitivity testing and molecular typing under my supervision. Other than the assistance outlined in the acknowledgements, the work described in this thesis is my own work and has not been submitted for a degree or other qualification to this or any other university.

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Abbreviations

AFLP	Amplified Fragment Length Polymorphism
ALT	Alanine Transaminase
AmB	Amphotericin B
AST	Aspartate Aminotransferase
BP	Blood Pressure
C	Celsius
CFU	Colony Forming Unit
CGB	Canavanine Glycine Bromothymol Blue
CI	Confidence Interval
CLSI	Clinical Laboratory Standards Institute
CNS	Central Nervous System
Cr	Creatinine
CrAg	Cryptococcal antigen
CSF	Cerebrospinal fluid
CT	Computerised Tomography
F	Fluconazole
FC	Flucytosine
GCS	Glasgow Coma Score
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
HR	Hazard Ratio
HTD	The Hospital for Tropical Diseases

IQR	Interquartile range
IRIS	Immune Reconstitution Inflammatory Syndrome
MIC	Minimum Inhibitory Concentration
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
N	Number
NA	Not Applicable
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NJT	Neighbour Joining Tree
OUCRU	Oxford University Clinical Research Unit
PCR	Polymerase Chain Reaction
Plat	Platelets
QC	Quantitative Count
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction fragment Length Polymorphism
TB	Tuberculosis
U	Units
ug	Microgram
uL	Microlitre
USD	United States Dollar
WCC	White Cell Count
WHO	World Health Organisation

Chapter 1
Introduction

Cryptococcus neoformans is an encapsulated basidiomycetous yeast (figure 1.1). The HIV pandemic has raised its profile from obscure fungus to the most important fungal cause of morbidity and mortality in the world. Previously best known as a rare cause of meningitis in patients with some form of acquired immunodeficiency such as haematological malignancy or organ transplantation, *Cryptococcus neoformans* is now estimated to cause 1 million cases of meningitis per year, and 720 000 deaths, most occurring within 3 months of diagnosis [1].

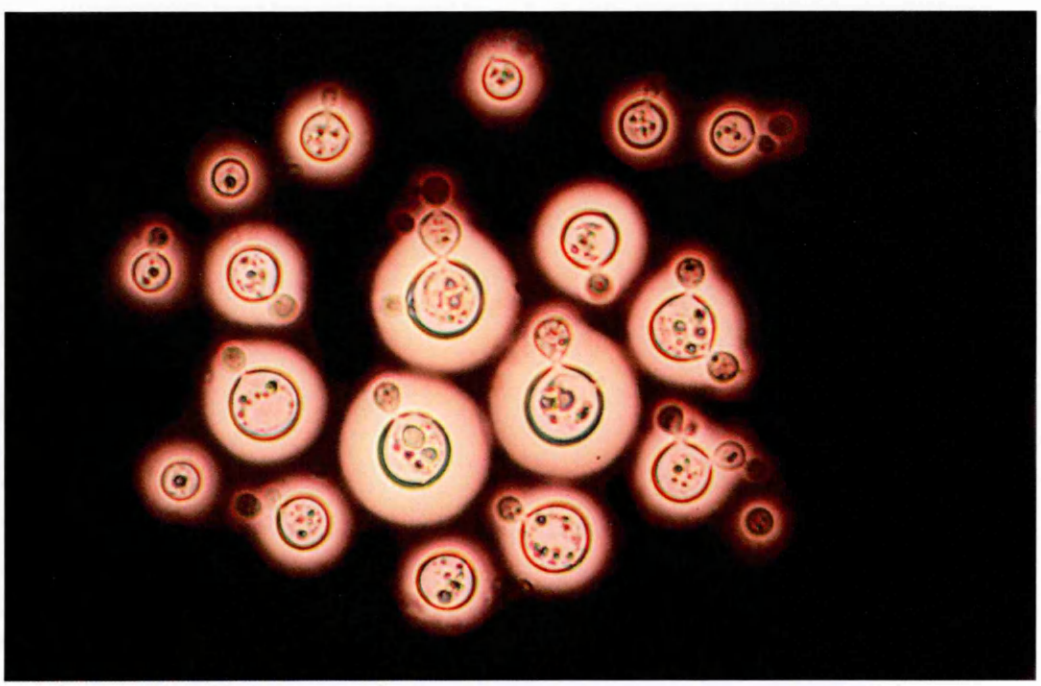


Figure 1.1 Encapsulated yeasts of *Cryptococcus neoformans* (India ink stain).

1.1 History

Studies on *Cryptococcus neoformans* began in 1894, when San Felice first isolated *Saccharomyces neoformans* from peach juice in Italy, and demonstrated that it was pathogenic in laboratory animals [2, 3]. He subsequently isolated an encapsulated yeast (which he termed *Saccharomyces lithogenes*) from the lymph node of an ox [4]. In the same year the first human infection was described by 2 German physicians, Otto Busse, a pathologist, and Abraham Buschke, a surgeon and dermatologist (who later perished with other intellectuals in the Theresienstadt concentration camp in 1943). They isolated an encapsulated yeast, which they termed *Saccharomyces hominis*, from the tibia of a 31 year old woman [4]. The second human case was described by Curtis in France in 1896 – he recovered a yeast-like fungus from a hip lesion and named it *Saccharomyces subcutaneous tumefaciens* [4]. Thus by the end of the 19th century the organism had been isolated from the environment, demonstrating that it could be free-living; from humans and animals, demonstrating its ability to cause disease; and it had been shown that it was possible to propagate the organism in the laboratory and infect laboratory animals.

In 1901 Vuillemin re-examined several of the isolates, and finding the ascospore characteristics of the *Saccharomyces* genus to be absent, reclassified the organism in the genus *Cryptococcus* [5]. *Cryptococcus hominis* was subsequently described as a synonym for *C. neoformans* [6]. During the first half of the 20th century numerous names were suggested for *C. neoformans*, including *Torula histolytica* (based on the mistaken assumption that it caused tissue lysis), *Cryptococcus histolytica* and *Debaromyces hominis*, but by the 1950s the term *Cryptococcus neoformans* had come

into widespread use and was quickly adopted by both the scientific and medical literature [7-9].

Through the beginning of the 20th century numerous case reports of human disease were published. The clinical syndromes were varied, including disease of the skin, tumour-like lesions, brain, bone, spine, lungs and kidneys. The first description of cryptococcal meningitis was published in 1905 by von Hansemann, although Freeman has argued that a case of chronic meningitis described in 1861 by Zenker, before the isolation of the organism, may represent the first case history [4, 10, 11].

1.2 Microbiology

The genus *Cryptococcus* now contains at least 39 species of yeast, but few are able to cause disease in humans [4]. Disease has rarely been attributed to other species such as *C. flavescens* (formerly *laurentii*) and *C. albidus*, but almost all human disease is due to infection with the *C. neoformans* species complex [12-15]. The *C. neoformans* species complex consists of *C. neoformans* var *grubii, C. neoformans* var *neoformans*, and *C. neoformans* var *gattii*. Since the middle of the last century, it was known that different isolates could be distinguished using rabbit sera. Evans and Kessel demonstrated 3 serotypes they termed A, B and C [16-18]. Subsequently a 4th serotype, D, was identified, and a hybrid AD strain discovered in the 1980s [19, 20]. In general, *Cryptococcus neoformans* var *grubii* and var *neoformans* type as serotype A or D respectively, and *Cryptococcus neoformans var gattii* as serotype B or C, although there are occasional inconsistencies reported in the literature [21].

The term *C. neoformans* var *gattii* was first used to describe an atypical strain of *C. neoformans* isolated from a leukaemic patient in 1970 [22]. *C. neoformans* var *gattii* has

recently been designated as a separate species (*C. gattii*) because of increasing data suggesting its distinctive evolution [23]. Using multigene sequence analysis it has been estimated that *C. gattii* diverged from *C. neoformans* approximately 49 million years ago [24]. *C. neoformans* and *C. gattii* can be distinguished by L-canavanine-glycine bromothymol blue agar (CGB), serotyping, RAPD patterns, PCR fingerprints, RFLP patterns, AFLP patterns and DNA sequencing [21, 25-35]. Additionally, most of these methods have shown that *C. neoformans* can be divided into 2 groups which have been described as separate varieties, a suggestion that was first made by Franzot based on both phenotypic and genotypic data [36]. These 2 groups are designated *C. neoformans* var *grubii* (serotype A) and *C. neoformans* var *neoformans* (serotype D). The 2 lineages are estimated to have diverged some 24.5 million years ago [24]. By far the majority of infections world wide are due to *C. neoformans* var *grubii*.

Cryptococcus neoformans and *C. gattii* are usually haploid yeasts that reproduce asexually[4]. However, they are dimorphic, and as well as existing in the asexual yeast form, characterised by oval to spherical cells with a polysaccharide capsule, have a sexual or perfect state which is characterised by the presence of basidiospores. Mating is a heterothallic process controlled by the MAT locus which determines the mating type (α or a), and consists of over 20 genes [6]. The MATα mating type is predominant in both clinical specimens and the environment for both *C. neoformans* var *grubii* and *C. neoformans* var *neoformans*, and is considered to be a virulence factor (being disproportionately prominent for *C. neoformans* var *neoformans* in clinical specimens) [37]. The MATa mating type of *C. neoformans* var *grubii* was thought to be extinct, and

has only recently been rediscovered [37]. Each mating type of *C. gattii* occurs in roughly 1:1 ratio. Interestingly the α mating type may play a role in neurotrophism [38].

The perfect state has not been described in association with clinical specimens and is observed only during mating, which has only been observed under laboratory conditions [6]. The asexual form reproduces through budding, which is frequently seen in clinical specimens. Some strains produce pseudohyphal forms that may be seen in tissue sections. Additionally, when under physiological stress, such as nitrogen deprivation or desiccation, *C. neoformans* can reproduce by haploid fruiting, a process which is similar to mating, although it is less effective in producing basidiospores, and is homothallic [6]. Haploid fruiting can occur with either mating type, but *C. neoformans* var *grubii* has poor fruiting ability, suggesting that it is not haploid fruiting that determines the different prevalences of the mating types within the environment [39]. Mating and haploid fruiting are clinically important since the infectious propagule is most likely the resultant basidiospores [40, 41].

C. neoformans is readily cultured in the laboratory, producing mucoid colonies within 36 to 72 hours on blood or Sabouraud's agar. Colonies are white to cream in colour, but characteristic dark brown colonies are formed when grown on birdseed agar (Figure 1.2a). The organism grows readily in automated blood culture systems. Identification can be confirmed through the demonstration of capsule growth on corn meal agar, development of characteristic brown mucoid colonies on birdseed agar, and through commercially available sugar assimilation test kits. Most strains are urease positive, turning urea agar pink (Figure 1.2b).

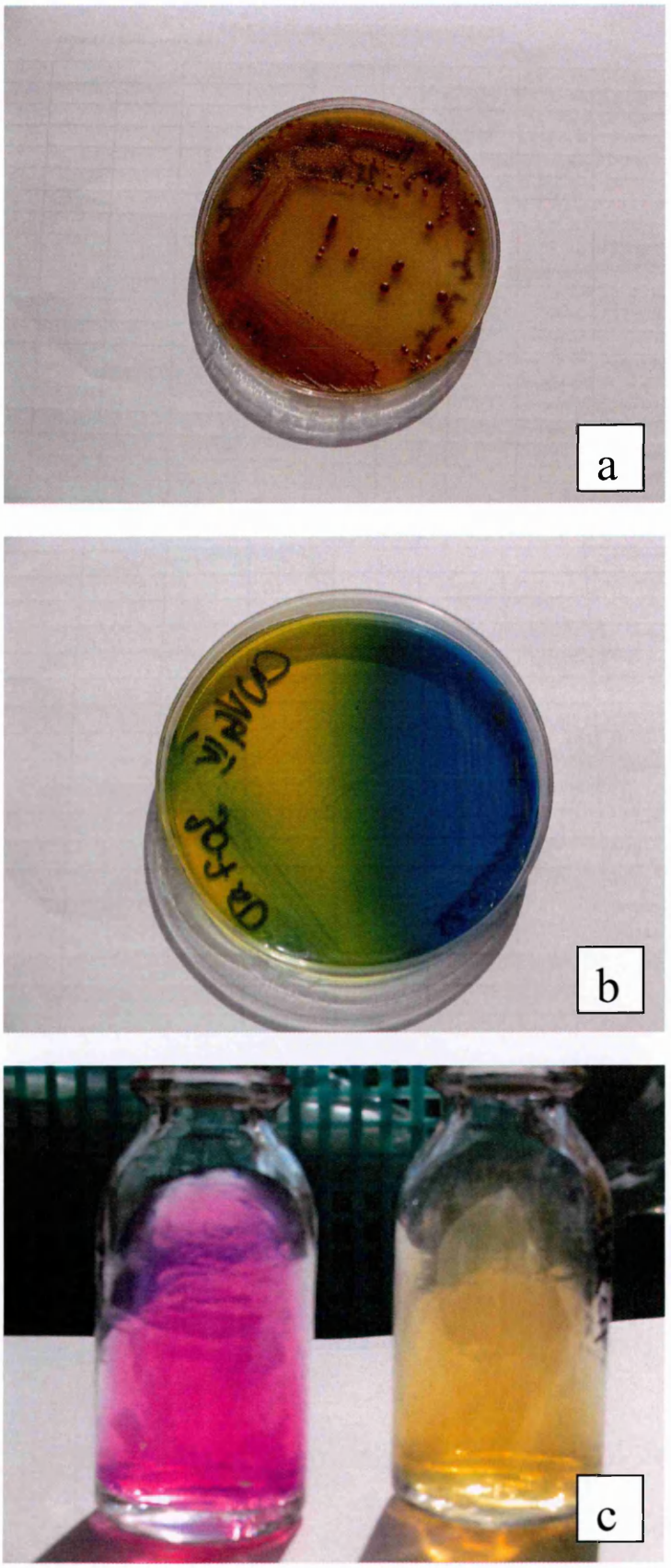


Figure 1.2 *Cryptococcus neoformans*

a) Characteristic brown colonies on bird seed agar

b) Blue discolouration of canavanine glycine bromothymol blue agar with growth of *Cryptococcus gattii*

c) Pink discolouration of urea agar due to urease production

Biotyping, to distinguish *C. gattii* from *C. neoformans*, can be done relatively cheaply using canavanine-glycine-bromothymol blue agar (Figure 1.2c).

1.3 Epidemiology

Neither *C. neoformans* nor *C. gattii* are primary pathogens, but are able to cause disease as a bystander effect of their adaptations to the environment. *Cryptococcus neoformans* is associated with pigeons, and has been repeatedly isolated from both the birds' crops and their droppings [42-45]. It has also been isolated from soil and decaying wood [46, 47]. *C. gattii* is associated with trees, first being associated with eucalypts, but has also been isolated from soil and associated with other tree species [48-50].

Antibodies to capsular antigen are ubiquitous in the sera of both HIV infected and uninfected asymptomatic individuals, suggesting that exposure to the organism is widespread [51]. The association with a bird as common and as widely distributed as the pigeon may explain *C. neoformans'* global distribution and the high rates of seropositivity. However, disease in otherwise healthy individuals is rare. In California the incidence rate of invasive cryptococcal disease in otherwise healthy people has been estimated at 0.2/million people/year [52]. Various conditions have been recognised to increase the disease risk, including sarcoidosis, lymphoproliferative disorders, hypogammaglobulinaemia, corticosteroid therapy, systemic lupus erythematosus, idiopathic CD4 lymphopenia, cirrhosis and peritoneal dialysis[4]. In particular, immunosuppression for solid organ transplant patients, and the use of monoclonal antibody based immunosuppressive therapies, are recognised and important iatrogenic risk factors for disease [53-55]. However, the major factor influencing the epidemiology of cryptococcal meningitis has been the HIV epidemic. Park et al have estimated the

global burden of disease due to cryptococcal meningitis, and conclude that the worldwide number of infections and deaths due to cryptococcal meningitis is similar to that reported for diseases that have achieved greater public health attention [1]. They estimated that there are almost 1 million cases of cryptococcal meningitis per year (range 370 000 – 1.44 million). As might be expected, most of these cases occur where there is little capacity to manage severe disease. There are estimated to be 720 000 cases annually in Africa, and South and Southeast Asia are the next most affected regions, with 120 000 cases per year. The number of deaths, derived from the case fatality rates reported for studies from the different regions, is estimated to be 625 000 per year (range 125 000 – 1 125 000). This approaches the number of deaths due to pertussis, poliomyelitis, diphtheria, measles and tetanus combined (530 000) [1, 56]. In Uganda, French reported twenty per cent of all AIDS deaths to be due to cryptococcal meningitis, making it the second most frequent cause of death in HIV infection after tuberculosis, similar to the experience at the Hospital for Tropical Diseases, Ho Chi Minh City [57, 58].

In addition, pulmonary cryptococcosis in HIV infected patients is probably under-diagnosed. An autopsy series of 8421 miners from South Africa (estimated HIV seroprevalence 24%) identified pulmonary cryptococcal infection in 7%. Only 1.9% of these cases had an ante-mortem diagnosis, being frequently misdiagnosed as tuberculosis (TB), bacterial or *Pneumocystis* pneumonia [59]. *Cryptococcus neoformans* may be responsible for more deaths than *Mycobacterium tuberculosis* in sub-Saharan Africa, recently estimated to account for 350 000 deaths per year [1, 56]. While most of these estimates were derived prior to the current efforts to improve access to anti-

retroviral therapy (ART), in fact rates of cryptococcal meningitis have actually increased in South Africa since the introduction of ART [1]. This could be a result of improvements in diagnostics, better case ascertainment, or immune reconstitution disease, all related to improved provision of HIV services.

Cryptococcus gattii tends to cause disease in the immunocompetent. In particular, disease is associated with male aboriginal people in northern Australia, and it is also an important cause of meningitis in Papua New Guinea [60-62]. The incidence of *C. gattii* infection may be increasing - while originally associated with tropical and sub-tropical regions, there has been a notable outbreak in British Columbia since 1999, and there is now evidence of autochthonous spread within the region [49, 63-69]. However, even in tropical regions where the HIV seroprevalence is high, infection with *C. gattii* remains uncommon [70]. As might be expected with a potential pathogen associated with eucalypts, disease in koalas is well described [71]. Disease described in wild dolphins associated with the British Columbia outbreak suggests that either *C. gattii* or dolphins are adapting their environmental niches in ways that have not been anticipated [72]! Despite their marine existence the route of infection in dolphins is likely the same as for other mammals – namely inhalation of air-borne spores.

Recently it has been reported that a large proportion of disease in immunocompetent Chinese patients is due to infection with *Cryptococcus neoformans* var *grubii*, and that these strains are genetically homogeneous. How they relate to strains isolated from HIV positive patients has not been elucidated.

At the Hospital for Tropical Diseases (HTD), Ho Chi Minh City, there has been a steady rise in the number of cases of cryptococcal meningitis since 1994, as illustrated by the

rates of its isolation from CSF in comparisons to *Streptococcus suis*, the commonest identified cause of pyogenic bacterial meningitis in our hospital (figure 1.3). This rise in cases parallels the AIDS epidemic in Viet Nam. *Cryptococcus neoformans* is now the most frequently isolated organism from both blood and cerebrospinal fluid at HTD. In addition, a small number of cases (2 – 10) of cryptococcal meningitis are seen each year at HTD in patients who are not HIV infected. Part of my work outlined in this thesis was aimed at speciating these strains and determining the relationship of these strains with those from HIV infected patients.

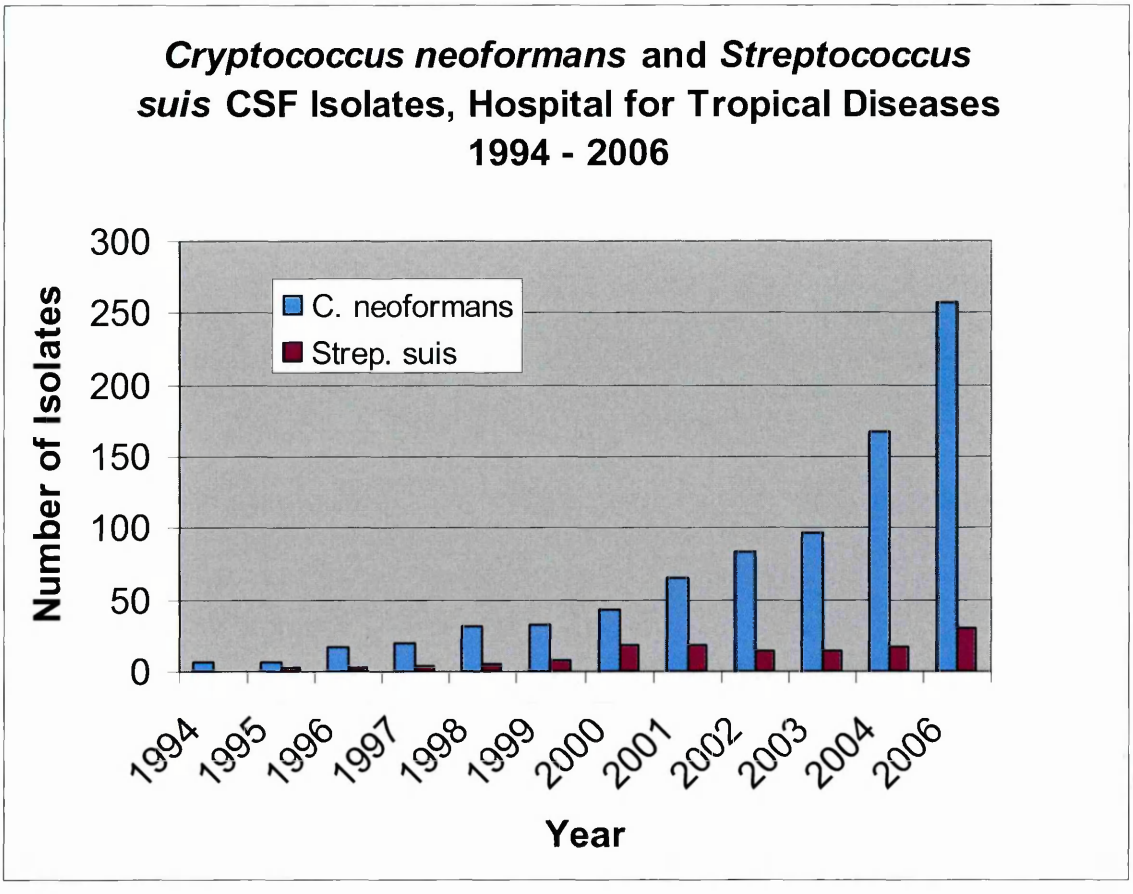


Figure 1.3 *C. neoformans* and *S. suis* isolates from CSF 1994 – 2006 at the Hospital for Tropical Diseases.

1.4 Human Disease

Infection of almost all organs has been described, but the primary organs of infection are believed to be the lungs, since this is the postulated route of infection. However, as the South African experience described above demonstrates, it is unusual for a diagnosis of pulmonary disease to be made in an HIV infected patient. It is not clear whether clinical disease is the result of recrudescence of latent infection, or the result of primary infection. The recent outbreak in British Columbia has allowed better estimates of the incubation period for *C. gattii*, because it has been possible to define the probable exposure periods for non-residents. The median incubation is estimated to be 6-7 months (range 2 to 11 months) [73]. It is more difficult to arrive at estimates for *C. neoformans*, since unlike *C. gattii* it is ubiquitous, and unlike tuberculosis spread is not via person to person. In France Dromer found that isolates from African patients were closely related, despite them having been resident there for a median of 9 years, suggesting that in HIV patients disease due to *C. neoformans* var *grubii* can be due to recrudescence of latent infection [74]. However, there are also reports of central nervous system (CNS) disease occurring short periods after recent exposure to pigeons and pigeon guano (with incubation periods as short as 7 to 10 weeks) [75].

1.4.1 Disease phenotypes

The major site of infection is the central nervous system (CNS), but disease of the lungs, skin, eyes, and prostate is also well described [4]. More rarely, infection occurs in bone, heart, liver, lymph nodes, joints, muscle, adrenal glands and kidneys [4]. Host immune status is thought to have a significant role in determining the disease phenotype, but

differences have also been ascribed according to the infecting species [4, 64, 76]. For example, focal neurological and pulmonary lesions, and visual impairment are more common with *C. gattii* infection [76]. However, by far the majority of cases of cryptococcal meningitis in HIV infected or uninfected patients are due to infection with *Cryptococcus neoformans* varieties. Significant co-morbidities are common in the HIV uninfected patients with cryptococcal meningitis, so the specific effect of HIV on clinical phenotype is not fully defined. The effect of HIV may be significant. Presence of a more robust immune response may increase the risk of residual disability, which may be reduced by specific adjuvant therapies [76, 77]. However, it is the results of treatment in HIV infected patients that are used to guide the treatment in HIV uninfected patients [78]. Better understanding the effect of HIV on the clinical phenotype of cryptococcal meningitis will help determine whether it s reasonable to extrapolate clinical trial results from one set of patients to another. In chapter 3 of the thesis I will examine the impact of HIV on the clinical phenotype of cryptococcal meningitis and discuss in more detail the current literature [4, 64]. A brief review of the clinical aspects of cryptococcal meningitis is presented below.

1.4.2 Clinical Features

Cryptococcal meningitis usually presents as a subacute meningitis, which is clinically indistinguishable from that due to tuberculosis [79]. Patients with HIV infection are most at risk when the CD4 count has fallen below 100cells/uL. Commonly reported symptoms include fever, headache, neck stiffness, malaise and visual disturbance. Visual loss seems to be particularly associated with *C. gattii* infection [80]. Nausea and

vomiting may indicate raised intracranial pressure. Physical examination may reveal other signs of severe immunosuppression, such as oral candidiasis and oral hairy leukoplakia, and signs characteristic of meningitis such as confusion, neck stiffness, papilloedema and cranial nerve palsies (figure 1.4).

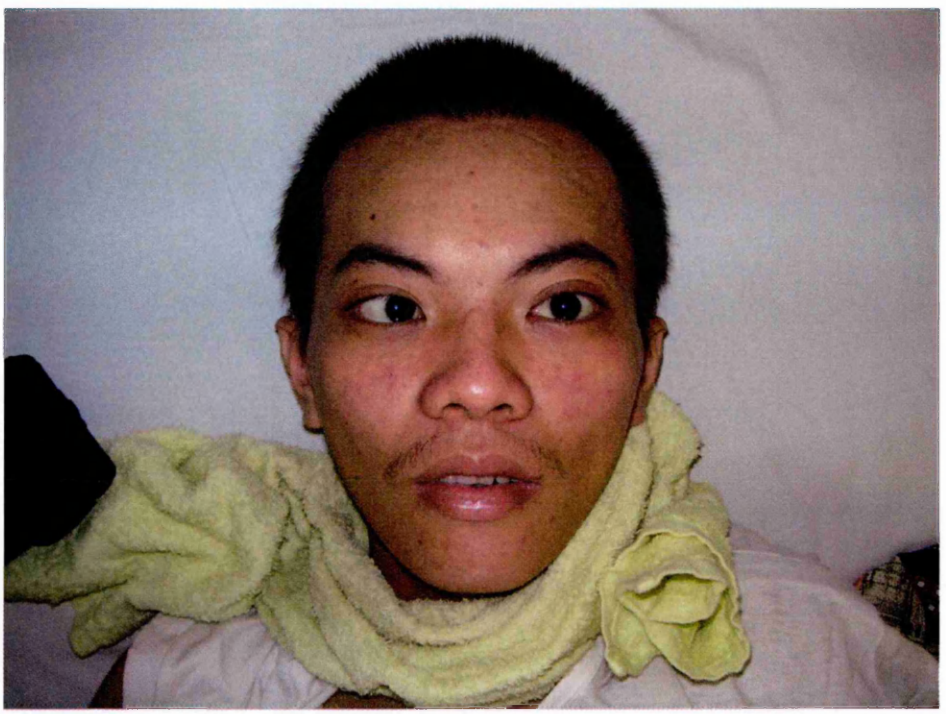


Figure 1.4 Left 6th nerve palsy in cryptococcal meningitis*.

Occasionally papular or ulcerated skin lesions are seen, which can be an important diagnostic aid where there are no facilities for microbiological confirmation (figure 1.5).

Figure 1.5 Typical skin lesion in cryptococcosis*.

However, in Asia *Penicillium marneffei* infection can cause a similar appearance (figure1.6).

Figure 1.6 Typical skin lesion due to *Penicillium marneffei*.

*all patients provided consent for photography

While HIV infection increases the risk of developing cryptococcal meningitis, the effect it has on the clinical phenotype is not clear. In tuberculous meningitis the death rate in HIV co-infected Vietnamese patients is 2-3 fold higher than in HIV uninfected patients [81]. Despite this increase in mortality, the neurological findings in patients with tuberculous meningitis with and without HIV co-infection are similar [81]. The influence of HIV on the presentation, course and outcome of cryptococcal meningitis is less clear, because of the relative rarity of cryptococcal meningitis in HIV uninfected patients, making clinical comparisons in single centres or even single geographical areas difficult. Moreover, a large proportion of cases of cryptococcal meningitis in HIV-uninfected patients are accounted for by patients with co-existing malignancies, organ transplants or other chronic diseases that are associated with morbidities and mortalities of their own, or are due to infection with *Cryptococcus gattii*, a species that probably accounts for less than 1% of all associated HIV infections worldwide [53, 70, 82-89]. As will be shown in chapter 3, the populations in whom cryptococcal meningitis are described are highly heterogeneous. Better understanding the particular effect of HIV infection on the disease phenotype of cryptococcal meningitis would allow the development of hypotheses regarding the pathophysiology of the disease in both HIV infected and uninfected patients (for example, which complications are immune mediated), and improve understanding of the generalizability of findings from one patient group to another.

1.4.3 Radiological Findings

Magnetic resonance imaging (MRI) has increased sensitivity in detecting intracranial abnormalities compared with computerised tomography (CT) brain scanning. Findings are not specific; tuberculous meningitis is the condition highest in the differential diagnosis. Hydrocephalus is the most common finding (figure 1.7) [90]. Basal meningeal enhancement may be present. Evidence of both parenchymal and meningeal disease can also be seen, but scans are frequently normal [91]. The abnormalities seen include cryptococcomas, dilated Virchow-Robin spaces and cortical nodules. The commonest parenchymal areas to be involved are the basal ganglia [91]. Cerebral infarctions, again usually seen in the basal ganglia but also affecting the thalamus and internal capsule, are indistinguishable from those seen in tuberculous meningitis [92]. Cryptococcomas are more common in immunocompetent patients, and usually enhance with contrast, although this effect can be attenuated in immunocompromised patients [90]. A miliary appearance has also been described [90]. Of note, at the Hospital for Tropical Diseases, we have seen patients with simultaneous culture confirmed cryptococcal and tuberculous meningitis.

Abnormal chest X-rays occur in up to one third of patients who present primarily with neurological disease. The manifestations are protean, varying from infiltrative lesions through to cavitation and mass lesions, particularly with *C. gattii* infection [4].

Figure 1.7 MRI scan showing hydrocephalus in cryptococcal meningitis.

1.4.4 Diagnosis

The definitive diagnosis is made through examination of CSF and the demonstration of encapsulated yeasts in cerebrospinal fluid, either through microscopy or culture. Microbiological diagnosis is considerably easier than in tuberculous meningitis - staining with India ink is usually sufficient, but cells are also easily seen with Gram's or Ziehl-Neelsen's stains [93]. *Cryptococcus* spp grow readily on blood and Sabouraud's agars although growth is enhanced for some strains at 30 rather than 35C. The CSF opening pressure is frequently raised, typically greater than 40mmCSF, and a mild lymphocyte predominant pleocytosis is characteristic, although CSF may be acellular, particularly in the immunosuppressed. The CSF to blood glucose ratio may be reduced

but can be normal. The protein is normal or raised. The CSF lactate is sometimes raised (typically 4-6mmols), but less so than is seen in pyogenic meningitis. In addition, cryptococcal antigen testing of CSF and serum is highly specific and sensitive, with sensitivities and specificities ranging from 83% to 97% and 93% to 100% respectively when compared to culture [94-96]. The sensitivity of the India ink test is in the order of 50% in non-AIDS and 80% in AIDS patients when compared to culture, the lower limit of detection being around 1,000 yeast cells/mL CSF, although under experimental conditions it performs similarly to antigen testing [97].

1.4.5 Prognostic factors

In tuberculous meningitis, well validated determinants of disease outcome (the United Kingdom Medical Research Council grading system) continue to apply well to HIV infected patients [98]. In cryptococcal meningitis, determinants of outcome are less clear. Individual studies have identified factors associated with outcome including fungal load at baseline, clinical features, presence of raised intracranial pressure and markers of inflammation in the CSF [61, 99-106]. However, these have rarely been tested in other datasets, investigators instead tending to identify further factors within their own cohorts. While identified prognostic markers may seem plausible, it cannot be discounted that the associations identified are simply chance findings [99, 101, 107, 108]. Treatment options for cryptococcal meningitis are limited, and have significant risks of toxicity. While treatment for all patients needs to be improved, the identification of reliable predictors of outcome on presentation may enable the identification of

patients in whom the use of intensified, but potentially more toxic, therapy can be justified [109].

In Chapter 3 of this thesis I discuss the literature on prognostic factors in more detail, and evaluate previously identified prognostic factors in a cohort of Vietnamese HIV infected and uninfected patients.

1.5 Treatment

Antifungal drug options for cryptococcal meningitis are limited. The Infectious Diseases Society of America has this year published revised treatment guidelines for cryptococcal meningitis [78]. They differ little in the recommended choice of antifungal regime from the guidelines published 10 years earlier [110]. The guideline authors suggest that the critical factors for successful treatment are host immunity, site of infection, drug toxicity and management of underlying disease. However, the lack of change in the guidelines perhaps also reflects the lack of trials that have been powered to clinical end-points in the last 15 years, which probably reflects the fact that most disease occurs in resource-poor settings. It seems surprising that in a disease where the median time to CSF sterilisation is in the order of 2 weeks, choice of antifungal therapy is not considered more critical [107].

1.5.1 Antifungals

1.5.1.1 Amphotericin B

Amphotericin B is the mainstay of treatment. This broad spectrum drug is fungicidal and *in vitro* resistance is extremely rare [111-115]. Amphotericin B causes membrane

disruption through binding to sterols in the cell membrane, but it probably also has an effect through stimulating macrophage function [116-118]. Bioavailability is extremely poor via the oral route and amphotericin B must be given intravenously [119]. The pharmacokinetics are poorly understood – it is difficult to detect in CSF but is effective in cryptococcal meningitis. Nephrotoxicity is a significant problem, although usually reversible if the total dose administered does not exceed 4g [119]. This is exacerbated by salt depletion, and a normal saline infusion is recommended prior to drug administration. In Vietnamese patients, anaemia is common (unpublished data). The lipid formulations of amphotericin B have the advantage of lower toxicities, and can be given in higher dosage of up to 10 mg/kg/day. However, they are considerably more expensive. An alternative route of administration is intra-thecally.

1.5.1.2 Flucytosine

Flucytosine is a nucleotide analogue. Available in oral and intravenous formulations, it appears to have a synergistic action with amphotericin in vitro [120]. Flucytosine is converted to fluorouracil within the fungal cell and inhibits RNA biosynthesis [121]. It probably directly inhibits DNA synthesis. A randomised trial showed a trend towards more rapid CSF sterilisation in patients receiving flucytosine in combination with amphotericin compared with amphotericin alone, but flucytosine has not been shown to reduce mortality in cryptococcal meningitis [107]. It has the disadvantages of high cost, poor tolerability, and rapid development of resistance if used as monotherapy. In wealthy countries monitoring of blood levels is usual.

1.5.1.3 Azole Drugs

The azole drugs have revolutionised the treatment of fungal infections because of their potency, tolerability, and oral and intravenous formulations. Fluconazole is the most frequently prescribed azole for cryptococcal meningitis. It has good CSF penetration, with peaks levels reaching 75-90% of serum levels, is relatively cheap and is widely available [122]. The mechanism of action of azoles is inhibition of sterol synthesis by the fungal cell and the effect is fungistatic [4]. Initial concerns that they might adversely affect the action of amphotericin if used in combination have not been born out in animal studies, and there are now data emerging from the treatment of systemic candidaemia that suggest this can be a powerful combination [123]. There is most experience with fluconazole in the treatment of cryptococcal meningitis. While it is not as potent as itraconazole in vitro, it has better CSF penetration and is more effective in clinical trials [107]. The newer azoles such as voriconazole and posaconazole appear to have better *in vitro* activity against *C. neoformans* than fluconazole, but there are no data from controlled trials in cryptococcal meningitis [124].

1.5.1.4 Echinocandins

These drugs, act by inhibiting 1,3-ß-D-glucan synthase complex and inhibiting cell wall synthesis [125]. They are generally fungicidal and active against azole resistant yeasts. Unfortunately, despite their otherwise broad spectrum, they have no activity against *Cryptococcus* spp.

1.5.2 Other Treatments

1.5.2.1 Interferon Gamma

There is direct *in vivo* evidence that interferon gamma levels are associated with the ability to clear yeast from CSF in HIV infected patients[126]. Pappas reported a small randomised trial of recombinant interferon gamma in 75 HIV patients and found improved fungal clearance rates in CSF at 2 weeks in patients receiving active drug, although the study was too small to detect differences in survival [127].

1.5.2.2 Corticosteroids

There has never been a randomised controlled trial of steroids in the treatment of cryptococcal meningitis. Dexamethasone is of proven benefit in tuberculous meningitis, although whether this is mediated through immune modulation is not clear [128, 129]. Potential mechanisms through which they could improve outcome in cryptococcal meningitis would include their anti-inflammatory effects and modulation of raised intracranial pressure. Their value in bacterial meningitis seems to vary by geographical location [130-132]. There are retrospective data suggesting that their use in *C. gattii* meningitis may reduce the incidence of visual complications and improve outcome[29, 31]. Seaton reported how the use of steroids (to treat reactions to amphotericin B infusion) was found to be highly associated with a reduced risk of development of visual loss or blindness (12.5% versus 70%, p=0.007 steroids versus no-steroids), and a 10-fold reduction in the risk of blindness (p = 0.018)[77]. In this study 13 patients received hydrocortisone (between 100 and 250mg/day), two received dexamethasone (16mg/day for up to 7 weeks) and one received prednisolone (7 weeks). One patient receiving

hydrocortisone improved, one patient receiving dexamethasone improved and the patient who received prednisolone improved. Two patients receiving hydrocortisone had deterioration in vision. The data reported by Seaton et al are weakened by the fact that they are retrospective and non-randomised, and that there was a change in the prescribing practice concerning steroids over time. However, they provide the best evidence yet of a potential disease modifying adjunctive treatment in cryptococcal meningitis. Their use in HIV patients in areas of high TB incidence would need to be carefully monitored, and a randomised controlled trial is needed.

1.5.2.3 Antiretroviral Therapy

It is not clear when to start antiretroviral therapy in HIV patients with cryptococcal meningitis. The potential benefits of immune reconstitution have to be balanced against the risks of drug toxicity and immune reconstitution inflammatory syndromes. Like tuberculous meningitis, most deaths in cryptococcal meningitis occur within the first month of treatment [81, 133]. A randomised trial of timing of initiation of antiretroviral therapy in tuberculous meningitis found no difference in mortality between starting 2 weeks after diagnosis and starting 8 weeks after diagnosis (Torok ME et al, unpublished data). A randomised controlled trial of early versus deferred antiretroviral therapy in 280 patients with a diverse range of opportunistic infections, 12% of which were cryptococcal disease, suggested that early introduction of ARVs in cryptococcal meningitis might be beneficial, but failed to reach conventional levels of statistical significance [134]. Conversely, in a retrospective review of almost 300 patients with cryptococcal meningitis in Thailand, Manosuthi found timing of initiation of

antiretrovirals had no impact on mortality (this dataset included 59 patients who began treatment within 1 month of diagnosis [135]. A recent trial from Zimbabwe on the timing of ARVs in cryptococcal meningitis was stopped early, ostensibly on the grounds of safety, but in fact had been struggling to recruit patients [136]. Immediate introduction of ARVs (within 72 hours of diagnosis) was associated with a worse outcome than delayed introduction (10 weeks). The median survival time was 28 days in the immediate arm versus 637 days in the delayed ARV arm (Hazard ratio 2.34, 95%CI 1.12 – 4.89), but the p value was only 0.031, a value much less significant that would usually be considered robust enough to stop a trial early.

1.5.3 Treatment for Particular Patient Groups

1.5.3.1 HIV Infected Patients

The current recommendations for HIV infected patients are that treatment is with amphotericin B 0.7 – 1mg/kg/day, in combination with flucytosine (100mg/kg/day) for at least 2 weeks (induction), followed by fluconazole 400mg/day for at least 8 weeks (consolidation). Following these 2 stages of treatment, patients require long term maintenance therapy with lower dose fluconazole until there has been a degree of antiretroviral therapy induced immune reconstitution.

These recommendations are derived from the large (380 patients) Mycoses Study Group randomised controlled trial published in 1997 [107]. This was a study carried out in the United States in HIV infected patients who, by nature of the study entry criteria, had relatively mild disease. Patients received amphotericin B in the dose described, and were then randomised to receive flucytosine or placebo. Despite the guidelines'

recommendation that flucytosine be included in the treatment, this study found no difference in outcome according to receipt of flucytosine. There was a non-statistically significant trend towards more rapid CSF sterilisation in the patients receiving flucytosine. A randomised controlled trial from Thailand compared the effect of 4 different antifungal regimes on the rate of clearance of yeast from cerebrospinal fluid (CSF) [99]. The rate of decline of viable yeast counts in CSF was greatest for the combination of amphotericin B with flucytosine, statistically significantly slower when combining amphotericin B with fluconazole 400mg/day or all 3 drugs together, and slowest with amphotericin alone. The dose of amphotericin B used was 0.7mg/kg/day, fluconazole 400mg/day and flucytosine 100mg/kg/day.

While the dosages of amphotericin B used in the Mycoses Study Group trial were in the range of 0.7 – 1mg/kg/day, the 'science' behind this original choice may have been that the drug is presented in a 50mg vial, and thus use of a single vial per dose for an average sized patient results in a dosage within this range. Recent data do suggest that significantly more rapid yeast clearance is achieved with a dosage of 1mg/kg/day versus 0.7 mg/kg/day [137]. The rate of decline of CSF yeast counts has subsequently been associated with outcome; these data are discussed in more detail in Chapter 5 [138].

It may be that the effect of flucytosine in combination with amphotericin is greater in more severe disease. Cohort data seem to support this – Dromer found that the treatment failure rate in patients receiving the combination of flucytosine and amphotericin was significantly lower than for patients receiving any other therapy, including amphotericin monotherapy (26% vs. 56%, $p < 0.001$) [139]. Patients receiving combination therapy tended to have more severe disease defined using conventional criteria such as the

baseline cryptococcal antigen titre and confusion. However, the presences of other markers of severe disease such as renal, liver or haematological failure, that may have precluded the use of flucytosine, and been associated with a worse outcome, are not mentioned in the study. amphotericin formulations have significantly reduced toxicities but much higher cost. In some patient groups, where the risk of renal impairment leading to dialysis is higher, it may be more cost effective, but this has not been rigorously tested in cryptococcal meningitis, and amphotericin B deoxycholate remains the recommended treatment [78, 140, 141].

Fluconazole monotherapy has been disappointing in cryptococcal meningitis [142-144]. One study examined escalating doses of fluconazole (800 – 2000mg/day with or without flucytosine) [145]. This 8 arm study recruited 88 patients, and patients considered to be more severely unwell (likely to die within 2 weeks) were excluded. The primary end point was treatment success, defined as surviving to 10 weeks with at least 1 negative CSF culture in that time period. The actual number of deaths is not described in the paper. The authors report an incremental increase in response as the dose of fluconazole increased, and the treatment success rate was higher for patients also receiving flucytosine. However, in many resource-poor areas it is the only treatment available. Itraconazole very poorly penetrates the blood brain barrier, and it has performed poorly in comparison with amphotericin when used as monotherapy, and in comparison with fluconazole when used for consolidation therapy [107, 146].

The value of fluconazole in preventing relapse in HIV infected patients who have completed treatment has been clearly demonstrated through a double blind randomized placebo controlled trial (which also demonstrated the prostate to be a potential site of

latent infection [147]. Alternative treatments are itraconazole or weekly amphotericin, but fluconazole has been shown to be superior in randomized controlled trials [148, 149].

Several studies have examined whether it is safe to stop maintenance therapy in HIV patients. If the patient has a sustained CD4 count of greater than 100 cells/uL on anti-retroviral therapy together and is symptom-free then stopping maintenance therapy is probably safe, although follow-up remains essential because there is still a real (but small) risk of relapse [150, 151].

1.5.3.2 Organ Transplant Patients

Around 2.8% of solid organ transplant recipients will develop cryptococcosis and the median time to disease onset is within 2 years [152]. Multi-organ involvement is frequent [78]. Because of the renal dysfunction associated with immunotherapy for organ transplant recipients, liposomal amphotericin is recommended [78]. Long term maintenance therapy with fluconazole is required - in its absence relapse approaches 100% within 1 year [153].

1.5.3.3 HIV Uninfected Patients

There are few data on which to base the treatment choices for this group of patients. The few randomised controlled trials which preceded the HIV epidemic had small numbers of patients, and used the low amphotericin B doses (0.3 – 0.4 mg/kg/day) that were consistent with the practice of the time [154, 155]. Furthermore, they preceded the arrival of the azoles, with their potential for tolerable long term therapy. The study of

Bennett is frequently quoted as the justification for the use of flucytosine in combination with amphotericin, although in this randomised controlled trial of amphotericin versus amphotericin plus flucytosine, there was no clinical outcome benefit on an intention to treat analysis [154]. Dismukes compared 4 versus 6 weeks of treatment with amphotericin and flucytosine in a randomized controlled trial, and found better response rates with the 6 week regimen. However, again the doses of amphotericin were lower than those used today (0.3mg/kg/day). The dose of flucytosine was 150mg/kg/day. Of note, the relapse rate was lower in the patients receiving the longer duration of therapy. Again, this trial preceded the azole drugs. Data from retrospective studies are suggestive that amphotericin and flucytosine are important in determining good outcomes, but are complicated by biases in determining treatment allocation [156, 157]. The updated treatment guidelines recommend that flucytosine combined with amphotericin (0.7 – 1mg/kg/day) for 6 weeks should be the basis of treatment for this group of patients [78]. This is probably safe, given that provided the total dose of amphotericin remains below 4g, any renal impairment tends to be reversible [119]. However, the value of adding flucytosine to this dose of amphotericin in immunocompetent patients has not been tested. Given a more robust immune response, its value may be limited.

No prospective studies have addressed the use of maintenance therapy in HIV uninfected patients. The relapse rate prior to the azole era was in the order of 15 – 25%, most occurring within the first year, and thus it is recommended that patients receive fluconazole maintenance therapy for 6-12 months [155].

1.5.3.4 *Cryptococcus gattii* meningitis

Cryptococcus gattii infections occur predominantly in immunocompetent hosts [60, 64, 76]. There is believed to be a lower response to therapy, perhaps as a result of walling off of foci of infection due to a more competent immune response. In addition, some reports suggest that *C. gattii* is inherently less sensitive to azole antifungals [158]. There has never been a randomised controlled trial of treatment of *C. gattii* infection. The treatment recommendations are essentially the same as for *C. neoformans* infection, but with increased vigilance recommended for the development of cryptococcomas that may benefit from surgical intervention.

1.6 Antifungal Sensitivity Testing

Numerous commercial antifungal susceptibility testing methodologies are now available for determining the susceptibilities of yeasts to currently available antifungals, and most systems have reasonable correlation with results of the standardised protocol (M27A) published by the Clinical and Laboratory Standards Institute (CLSI) [159-169]. Minimum inhibitory concentrations of antifungal drugs have been shown to have reasonable correlation with outcome in infection with *Candida* species, depending upon the site of infection and drug tested. In a murine model, outcome of treatment with azoles is related to *in vitro* azole susceptibility [170]. In humans with *Candida albicans* mucosal infection, breakpoints have been derived for fluconazole, itraconazole, and voriconazole that are based upon the outcome of treatment [171-173].Testing the susceptibility of isolates to fluconazole has been demonstrated to correlate with the clinical outcome of mucocutaneous disease in HIV infected patients, and thus has

clinical utility [174-176]. However, no relationship between MIC and clinical outcome has been demonstrated for caspofungin and oesophageal candidiasis, although this is an effective treatment [177]. There has been limited success in correlating antifungal susceptibilities with the outcome of candidaemia. A relationship has not been demonstrated for amphotericin B, the key therapeutic agent [178]. However, there is probably a role for azole testing in this situation, where a relationship between fluconazole MICs and outcome has been demonstrated in more than one study in HIV uninfected patients [179, 180]. A novel method for determining susceptibility to fluconazole using flow cytometry has been shown to correlate with outcome of *Candida* fungaemia in one small study [181]. Interestingly, in this small study of 24 patients, there was reasonable correlation between the flow cytometric methodology and results obtained using the CLSI protocol, but only the results obtained using flow cytometry correlated with clinical outcome.

There are fewer data for cryptococcal disease, and a relationship between the antifungal MICs and outcome has not been consistently demonstrated [114, 161, 182, 183]. *Cryptococcus* spp with reduced sensitivity to antifungals have been detected in the environment; additionally, the long term use of azole prophylaxis has been associated with the development of resistance amongst *Candida* isolates, and given the evidence that cryptococcal disease may represent reactivation of latent disease, there may be the potential for the selection of resistant strains within patients [158, 184]. Currently, the American Infectious Diseases Society does not recommend the use of antifungal susceptibility testing in first presentations of cryptococcal disease. This is because it is not well validated, and detectable resistance at initiation of therapy is uncommon, at

least in the USA [78, 185]. In Chapter 5 of the thesis I investigate the antifungal susceptibilities of Vietnamese *Cryptococcus* isolates, relate this to rates of fungal clearance and outcome and discuss the literature in more depth regarding antifungal susceptibility testing in relation to cryptococcal meningitis.

1.7 Complications

1.7.1 Raised Intracranial Pressure

Raised intracranial pressure is a frequent feature of cryptococcal meningitis in both HIV infected and uninfected patients, present at baseline in approximately half of all HIV patients and 80% of HIV uninfected patients with *C. gattii* [62, 186]. Raised intracranial pressure seems to be related to yeast burden, and interestingly in one small study was not related to the levels of pro-inflammatory cytokines [186]. Management of raised intracranial pressure is believed to be crucial, although Seaton did not find it to be associated with outcome in *C. gattii* infection [62]. There has been only one randomised controlled trial of an intervention to control pressure. This trial investigated the utility of acetazolamide, a carbonic anhydrase inhibitor which reduces CSF production [109]. Unfortunately the study had to be stopped early because of an excess of deaths in the intervention arm, due to metabolic acidosis, considered to be a consequence of renal tubular acidosis induced by the drug. However, the dose of acetazolamide was relatively high compared with that used to treat and prevent acute mountain sickness. Raised intracranial pressure often develops during the course of treatment, and acetazolamide may be a useful therapy either in lower dose, or after the induction phase of treatment. Further trials are needed. Other possible drug treatments include mannitol or

corticosteroids, but there are no data to support their use. Repeated drainage by lumbar puncture is advocated by most clinicians.

There is limited experience with the use of lumbar drains. The main concern is the introduction of infection, although the rate was reported to be less than 5% in one case series of bacterial meningitis [187]. A drain was used for 13 days without problem in one case report [188]. Where there is access, the insertion of ventricular-peritoneal shunts has been used to manage chronic hydrocephalus. Provided the patient is receiving antifungal therapy, insertion during acute infection seems to be safe, but the outcome is worse if the Glasgow coma score is less than 9 at the time of insertion [189-191].

1.7.2 Visual Loss

Visual loss is well recognized in cryptococcal meningitis, and appears to be more common in the immunocompetent than in HIV patients [192, 193]. The mechanism of damage is not clear, but lesions all along the optic tract have been described. Some studies have found visual loss to be associated with raised intracranial pressure but this relationship is not always present. Direct optic nerve invasion by cryptococci has been demonstrated in post-mortem specimens. Other mechanisms suggested include optic nerve compression by inflammatory adhesions, endophthalmitis and cryptococcomas [80]. The difference in rates of visual loss between HIV positive and negative patients, along with the potential disease modifying effect of steroids, lend weight to the hypothesis that the damage is in some way immune mediated.

1.7.3 Other focal neurological deficits

The most common sequelae in survivors of cryptococcal meningitis are visual impairment and hearing loss [62]. The commonest focal neurological deficit is a sixth nerve palsy, usually indicating raised intracranial pressure. As in tuberculous meningitis cerebral infarction, perhaps due to associated vasculitis, is described. This can occur in any vascular territory but characteristically affects the basal ganglia [194]. Disability at the end of treatment, with the exception of visual loss, is infrequently described in the literature suggesting that these events are uncommon.

1.7.4 Relapse

The major clinical question in patients who relapse is how to effectively retreat the patient. There have been no randomised controlled trials of salvage therapy, but there is some experience of using later generation azoles, (voriconazole and posaconazole) in this situation [78]. The success rates with these agents in salvage is in the order of 40% [78]. Fluconazole resistance is associated with treatment relapse, and cross-resistance is likely between different azoles, so this low rate of success might be expected [195].

1.7.5 Immune Reconstitution Inflammatory Syndrome (IRIS)

The development of effective antiretroviral therapy has been associated with the emergence of IRIS as a complication of HIV disease, although a similar immune phenomenon has been well recognised in tuberculosis for some time [196]. IRIS describes a condition whereby a disease syndrome arises or deteriorates not directly due to treatment failure, but due to a rapid improvement in the immune responsiveness of the

host with resultant inflammation. Usually the pathogen in question is not culturable at the time of IRIS. The difficulty is in distinguishing IRIS from treatment failure. Currently there is no diagnostic test for IRIS, and it remains a clinical diagnosis. The incidence of IRIS in cryptococcal meningitis is in the order of 13% [197]. Identified risk factors include low CD4 count, no previous anti-retroviral treatment, positive CSF cultures at 2 weeks , and rapid response of viral load to therapy [198]. IRIS occurs not only in HIV patients, but also in patients with organ transplantation and the apparently immunocompetent [78]. As well as presenting as meningitis, it can present in a body tissue that was not the initial site of disease presentation [199, 200]. IRIS can present any time from a few weeks after initiation of therapy to several months. The consequences of IRIS can be severe, but there are no randomised controlled trials to guide management. For IRIS affecting the CNS, steroids are suggested [78]. Retrospective data suggest that cryptococcal antigen testing of the serum of patients beginning ARV therapy can identify patients at risk of developing cryptococcal disease within the first year of treatment [201]. Some of this disease may be IRIS, and it is plausible that an antifungal intervention might reduce this risk. A trial is needed.

1.8 Outcome

The outcome in cryptococcal meningitis varies profoundly according to geographical location [1]. The mortality rate varies between 100% at 6 months in sub-Saharan Africa, to around 10% at 10 weeks in well resourced settings [107]. This is almost certainly influenced by the ability to make a timely diagnosis, access to treatment other than fluconazole, and the ability to manage complications and raised intracranial pressure

[78]. In southeast Asia, the mortality rate is in the order of 40%, and most deaths occur within the first month of treatment [202]. The advent of effective antiretroviral therapy has done little to impact on the early mortality in cryptococcal meningitis, but the long term mortality for patients who survive meningitis and begin antiretroviral therapy has improved dramatically [203]. This remains true for patients in less well resourced countries [137, 204, 205]. In most infectious diseases, early diagnosis and instigation of effective treatment is key in determining a good outcome, and the same is likely to be true in cryptococcal disease. One tool that may be useful in identifying patients at risk of cryptococcal disease is serum cryptococcal antigen testing. It has been shown that asymptomatic cryptococcal antigenaemia precedes the onset of disease, and is an independent predictor of mortality in patients in Uganda [206]. Micol found 11% of asymptomatic first time attending HIV patients in Cambodia to have positive serum cryptococcal antigen tests [207]. All patients were treated in this study, so the natural history of antigenaemia in this group is not known. Whether or not asymptomatic antigenaemia should be treated is not clear, but it identifies a group of patients in whom heightened surveillance for cryptococcal disease is prudent. Primary prophylaxis for cryptococcal meningitis with azole drugs has been investigated in randomized controlled trials. While it seems to reduce the risk of invasive fungal disease, in the absence of antiretroviral therapy it does not seem to be associated with a mortality benefit [208-210]. Furthermore, long term use of itraconazole as prophylaxis has been associated with reduced azole susceptibilities of *Candida albicans* strains in HIV patients, and thus azole primary prophylaxis is generally not recommended, although it forms part of the national guidelines in both Thailand and Viet Nam [211].

1.9 Summary

Thirty years ago the idea that meningitis due to a yeast would be amongst the leading infectious causes of death in adults would have been met with incredulity. If Park and colleagues are correct, then *Cryptococcus neoformans* is a more frequent cause of death in adults in sub-Saharan Africa than tuberculosis. Cryptococcal meningitis is relatively easy to diagnose, but the high mortality rates illustrate the difficulties in its treatment. The arrival of the azole drugs, with their tolerability and ease of administration, has had an important impact on the ability to manage this disease in the long term, but when used as monotherapy their performance has been disappointing, perhaps reflecting the fungistatic nature of their action. The mortality rate remains high, particularly in poorly resourced countries, and there has not been a major advance in treatment since 1997 [107]. Effective treatment still relies on 2 drugs, amphotericin B and flucytosine, that are over 50 years old, have significant rates of side effects, and are expensive. In addition, they are poorly available where most disease occurs.

Despite large numbers of patients, and a high frequency of endpoints (mortality rate), there is already a move away from clinical endpoint trials in cryptococcal meningitis, perhaps because of cost and the pressure to publish [99, 137, 138, 186, 205]. Measuring the rates of fall of viable yeast counts in CSF seems to be a sensitive detector of the potency of antifungal efficacy, but surrogate markers can be misleading. Surrogate markers, such as early fungicidal activity, are unable to detect effects of treatment on mortality that are not related to the expected mode of action. Reliance on surrogate markers can thus hinder therapeutic advances. For example, one of the few HIV antiretroviral studies powered to clinical endpoints (the SMART trial of structured treatment interruptions) gave results that few would have predicted prior to the study,

and has led to new insights into the immunopathogenesis of cardiovascular disease [212].

Because cryptococcal meningitis in immunocompetent patients is rare, and the most studied disease in immunocompetent patients has been due to infection with *C. gattii*, the disease modifying effects of HIV are not as clearly defined as might be expected. In China, disease in the immunocompetent has been reported to be due to *C. neoformans* var *grubii*, the variety responsible for most HIV related disease worldwide. Comparing the clinical phenotypes between these 2 groups of patients would enable a determination of whether disease phenotype is primarily driven by the immune response or the pathogen itself, and is a question I will address in this thesis.

A large number of prognostic factors have been identified in patients with cryptococcal meningitis. This contrasts with tuberculous meningitis, where a robust grading system exists that correlates well with outcome in both HIV infected and uninfected patients. The infectious disease research community has a poor record in performing large adequately powered clinical trials. This is in marked contrast to the history of clinical research in cardiovascular diseases [213, 214]. A problem with smaller randomised controlled trials can be the unequal distribution, by chance, of patients with different severities of disease between treatment arms. This can be ameliorated by stratification according to disease severity. If robust prognostic factors can be identified in cryptococcal meningitis the practical uses would include stratification in clinical trials. In addition, the prognostic factors could be used to identify patients who may need more intensive treatment, or in whom more toxic but potentially more effective treatment could be justified. The already identified prognostic factors in cryptococcal meningitis

need to be evaluated in an independent dataset, and this will form part of the work of this thesis.

Cryptococcal meningitis in HIV uninfected patients is rare. When it occurs, the question arises as to whether it is due to some undiagnosed immune deficit in the patient, or is due to contact with a rare strain of *Cryptococcus* that has increased virulence. I will address this question in the thesis through investigating the molecular epidemiology of strains in Viet Nam.

Finally, there are no data on the antifungal susceptibility of *Cryptococcus* spp from Viet Nam. While antifungal sensitivity testing has been shown to have some clinical utility in *Candida* infection, this has not been shown for cryptococcosis, although the studies are generally small. In this thesis I will describe the antifungal sensitivities of Vietnamese isolates and relate these to *in vivo* responses and clinical outcome.

Chapter 2

Materials and Methods.

2.1 Introduction

This chapter describes the research setting, region, hospital and study wards, and outlines the focus, aims and structure of the thesis. General clinical research and laboratory methods are described. Further relevant details are also described in subsequent chapters.

2.2 Setting

2.2.1 Geography

Viet Nam has a population of 86 million people, is the world's 13th most populous country and fifty per cent of the world's population lives within 2000km of Ho Chi Minh City. The land mass occupies 329 500 square km and stretches over 1600km along the eastern coast of the Indo-Chinese peninsula, lying between latitudes 8^{o} and 24^{o} north of the equator (figure 2.1). Subsequently there are wide variations in climate along the length of the country, the tropical south having hot dry winter months followed by the monsoon season (May to November). In the north, the capital Ha Noi has a temperate climate. In recent years the country has seen rapid economic growth of 6.5 - 9% p.a. [215]. Gross national income per capita in 2008 was 890USD/year, but 21% of the population live on less than 1.25USD/day [216]. The country received 1.86 billion USD in overseas development aid in 2006, and 4% of government expenditure is spent on health [216].

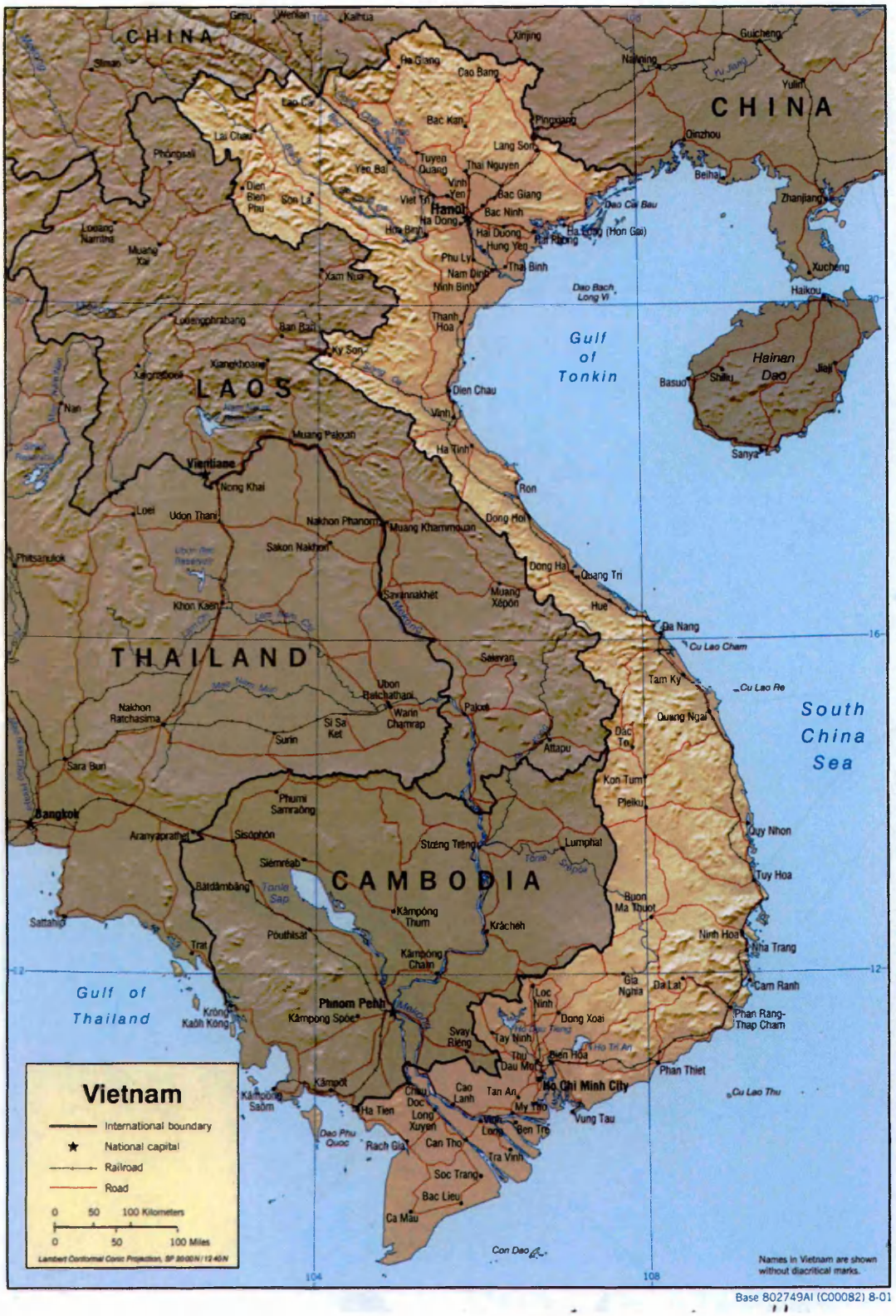


Figure 2.1 Map of Viet Nam (www.worldmapfinder.com)

2.2.2 Health Service

Health care is delivered by the private and state sectors. Most hospitals are state owned and run. WHO estimated there were 6 doctors per 10 000 of the population in 2002 (more recent data are unavailable) and government spending on health per capita was 10 USD in 2005, accounting for 6% of gross domestic product. Limited health insurance is provided by the state for all legally resident children up to the age of 6 years, but all other health costs must be borne by the patient, with the exception of chemotherapy for tuberculosis. State employees and employees of foreign-owned companies have health insurance, although the cover provided is limited. There are well developed and efficient referral patterns from commune health posts to district hospitals, onward to provincial hospitals and finally to tertiary referral centres such as the Hospital for Tropical Diseases. As a consequence, the Hospital for Tropical Diseases has a catchment population of 40 000 000 people. Life expectancy at birth is currently 74 years, the under-5 mortality rate is ranked 126 in the world at 15 per 1000 and HIV prevalence is estimated at 0.5% [215, 216].

2.2.3 The Hospital for Tropical Diseases, Ho Chi Minh City

The Hospital for Tropical Diseases (HTD) acts as a primary, secondary and tertiary referral centre for patients with infectious diseases from the whole of southern Viet Nam. It has approximately 500 beds and admits 35 000 patients per year. There are paediatric and adult intensive care wards. Important causes of admission include HIV, hepatitis, dengue fever, tetanus, pneumonia and septicaemia. The Viet Anh ward has 18 beds with a dedicated 5 bed intensive care unit where ventilation and haemofiltration can

be performed. HTD has laboratories for haematology, biochemistry, microbiology, serology, parasitology and mycology. These facilities are housed in the clinical sciences building alongside the OUCRU. Hospital laboratories partake in an external quality assurance scheme organised through the South East Asia Infectious Diseases Network (www.seaicrn.org).

2.2.4 Oxford University Clinical Research Unit

OUCRU was established in 1991, and historically has been primarily funded by the Wellcome Trust UK. Its purpose is to facilitate high quality clinical and laboratory-based scientific research and training in order to improve human and animal health. The research interests of the Unit are driven by the clinical needs of the local population, and initially work was focused on severe malaria[217-220]. Over the past 18 years the research interests have expanded to include central nervous system infections, dengue, respiratory infections, tetanus, enteric infections, helminthiases, tuberculosis and HIV[81, 129, 130, 221-241]. Research trials are run on multiple wards within the hospital, including the Viet Anh ward (formerly the Malaria Ward and now almost exclusively concerned with CNS infections), the adult intensive care unit, the paediatric intensive care ward and a general infectious diseases ward.

2.3 HIV in Viet Nam

There were an estimated 243 000 people infected with HIV in Viet Nam in 2009 and the prevalence doubled between 2000 and 2005 [242]. HIV cases have been reported from all 64 provinces/cities. The prevalence in 15 – 49 year olds is estimated at 0.5% (95%

confidence intervals 0.3% to 0.9%), which is similar to Spain, although the epidemics are at different stages [242]. The prevalence is rising in Viet Nam, expected to increase by 10% by 2012, with around 12 000 new infections per year [243]. The epidemic is currently concentrated among key populations at higher risk including intravenous drug users, sex workers and their partners, and men who have sex with men, but heterosexual transmission is becoming more significant [243]. Ho Chi Minh City has the highest number of reported cases of HIV (23 231 reported by July 2006) [242]. In 2007 28.4% of adults with advanced HIV infection were believed to be receiving anti-retro viral therapy (ARV), and only 13% of pregnant women received ARVs to prevent mother to child transmission[242].

At the Hospital for Tropical Diseases the number of patients admitted with HIV infection has risen dramatically since 1994. The total hospital mortality rate for all in patients was falling until the mid 1990s, but since then has risen, and this increase in death rate is entirely accounted for by HIV infection. 75% of all in-hospital deaths are now accounted for by HIV infected patients. In concert with this rise in HIV prevalence, *Cryptococcus neoformans* has become the commonest microbiological isolate from both cerebrospinal fluid and blood (Figures 2.2 and 2.3).

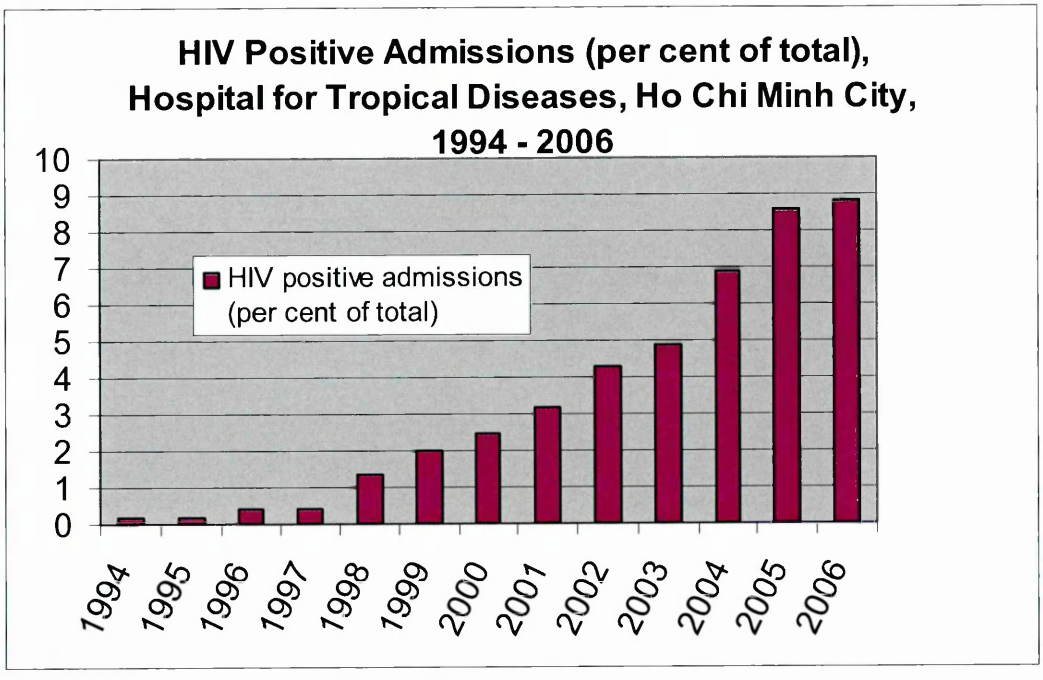


Figure 2.2 HIV admissions at HTD as percentage of total 1994 - 2006

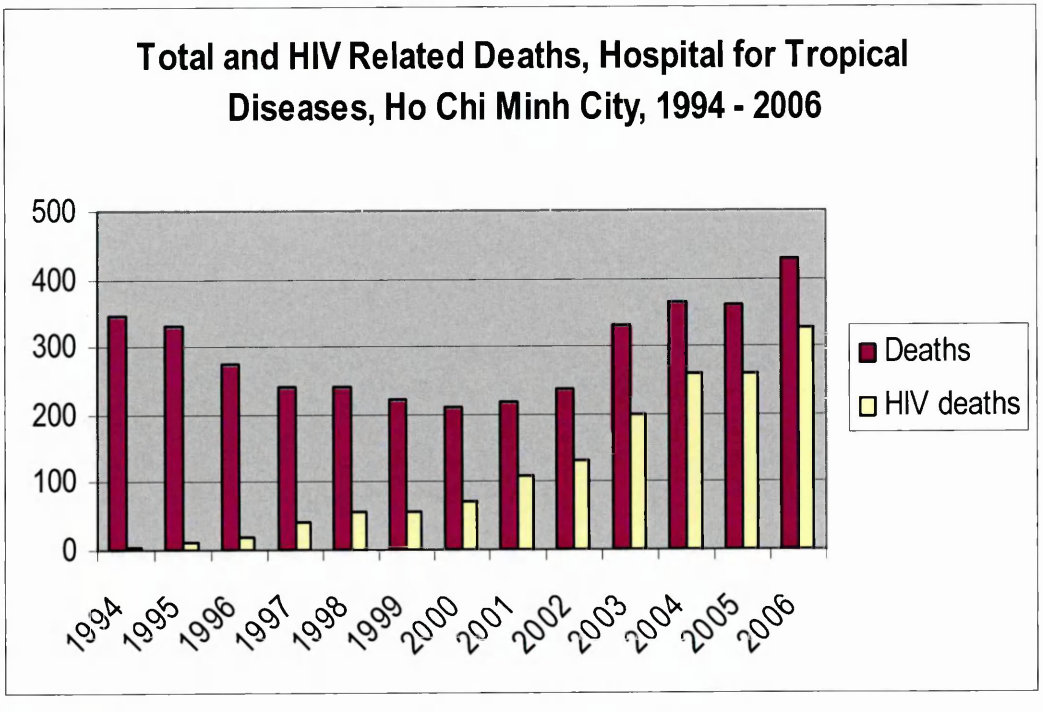


Figure 2.3 HIV in-patient deaths at HTD, as per cent of total 1994 - 2006

2.4 Focus, Aims and Structure of the Thesis

This thesis examines cryptococcal meningitis in Viet Nam, and aims to address the following questions:

1. Is the clinical phenotype of cryptococcal meningitis influenced by HIV infection?
2. What are the prognostic factors in cryptococcal meningitis?
3. What is the molecular epidemiology of human isolates of *Cryptococcus neoformans* in Viet Nam, and is this influenced by HIV serostatus?
4. What is the utility of antifungal sensitivity testing in cryptococcal meningitis, has the antifungal susceptibility of *C. neoformans* changed over time, and do the susceptibility profiles of *C. neoformans* differ by infecting variety and immune status of the host?

These questions are addressed in the following chapters.

2.5 Clinical Methods

2.5.1 Scientific and Ethical Approval

HTD Scientific and ethical committee approved all study protocols, and informed consent was obtained from each patient or accompanying relative. In addition to ethical approval from the Hospital for Tropical Diseases, the BMD study had ethical approval from the Oxford Tropical Ethics Committee and the BK study from Liverpool School of Tropical Medicine Research Ethics Committee.

2.5.2 Study Site

All studies took place at the Hospital for Tropical Diseases, Ho Chi Minh City. All patients with suspected or definite central nervous system infections are referred to the Viet-Anh (Clinical Research Ward) where they are assessed and cared for by a dedicated Clinical Research team of doctors, nurses and allied professionals. All patients in this analysis were recruited to one of 2 studies – the BMD study or the BK study (the study codes BMD and BK have no specific meaning but were codes allocated from an internal trial registry spreadsheet). HIV uninfected patients were recruited from the BMD study, and HIV infected patients from the BK study.

2.5.3 The BMD Study

This study is an on-going prospective descriptive study of HIV uninfected patients with central nervous system infections at the Hospital for Tropical Diseases. Informed consent is obtained from all patients. All HIV uninfected adults ($\geq$ 15 years old) with a syndrome consistent with a central nervous system infection, or a recognized pathogen isolated from cerebrospinal fluid, are eligible for study entry. Other than HIV infection, there were no exclusion criteria. Thus the HIV negative cryptococcal meningitis patients form a subset of patients from this study. All patients were recruited consecutively from between 1996 and 2007. The definition of cryptococcal meningitis was any patient presenting with CNS symptoms (e.g. fever, headache, neck stiffness, confusion, coma, convulsions, focal neurology) and a positive cryptococcal antigen test, India ink or culture of *Cryptococcus species* from either cerebrospinal fluid (CSF) or blood, or any patient with positive India ink, cryptococcal antigen titre or culture of *Cryptococcus*

species from CSF in the absence of CNS symptoms. Evidence of any other central nervous system infection was an exclusion criterion. All patients had to have 2 negative HIV antibody/antigen and negative rapid tests (HIVAXSYM HIV1/2gO, Determine HIV1/2, Abbott, Maidenhead, UK).

2.5.4 The BK study

The BK study is an ongoing 3 arm open label randomized controlled trial of antifungal treatment for cryptococcal meningitis in HIV infected adult patients. All patients for this analysis were taken from the control arm, in which patients receive treatment for cryptococcal meningitis according to Vietnamese guidelines. The definition of cryptococcal meningitis was a syndrome consistent with meningoencephalitis and isolation of *Cryptococcus* species (culture or positive cryptococcal antigen titre from blood or CSF or positive India ink from CSF), or isolation of *Cryptococcus* species from CSF in the absence of neurological symptoms. Study recruitment began in April 2004. The exclusion criteria were a previous diagnosis of cryptococcal meningitis, previous azole therapy, pregnancy or renal or liver failure, or active TB requiring treatment with rifampicin.

2.5.5 Clinical Assessment

All patients in both studies had a full clinical evaluation at study entry including detailed history and physical examination by the dedicated study team.

2.5.6 Investigations

Routine haematological investigations and CSF examination using standard methods were performed on all patients at study entry including full blood count, urea and electrolytes, liver enzymes, blood glucose, CSF cell counts, protein and glucose. CSF samples were stained and cultured by standard methods for pyogenic bacteria, fungi, and mycobacteria. Opening CSF pressure was measured at each lumbar puncture. Cryptococcal antigen titres were determined using the Remel *Cryptococcus* Antigen Test Kit according to the manufacturer's instructions (Lenexa, USA). All patients were tested for antibodies to HIV (Determine HIV1/2; Abbott), and positive results were confirmed by Western blot analysis. For all HIV-infected adults, peripheral blood CD4 and CD8 lymphocyte counts were measured as soon as possible after randomization by use of flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, USA).

2.5.7 Imaging

Chest X-rays and brain imaging (Computerised Tomography (CT, Toshiba XVIsion, Toshiba, Japan) or Magnetic Resonance Imaging (MRI, General Electric, UK, 1.5T)) were performed at the physician's discretion.

2.5.8 Treatment

All HIV-infected patients received treatment with amphotericin B 1mg/kg/day (Photericin B®, Cipla Ltd, Mumbai, India) for 4 weeks followed by fluconazole 400mg/day (Flucoric®, Ranbaxy Laboratories Ltd, Gurgaon, India) for a further 6 weeks. The fluconazole dose was then reduced to 200mg daily as long term secondary

prophylaxis. Antiretroviral therapy was prescribed at the attending physician's discretion. All patients received *Pneumocystis jirovecii* primary prophylaxis with 960mg trimethoprim-sulfamethoxazole daily.

HIV negative patients were also treated with amphotericin B 1mg/kg/day for at least 2 weeks, prior to switching to fluconazole. The duration of therapy with amphotericin B beyond this point was at the attending physician's discretion. Flucytosine 100mg/kg/day, in 4 divided doses (Ancotil®, Valeant Pharmaceuticals International, CA, USA) could be given for the first 2 weeks at the physician's discretion. Following amphotericin patients were switched to fluconazole 400mg/day.

2.5.9 Management of raised intracranial pressure

All patients had the opening CSF pressure measured using a spinal manometer. The upper limit of measurement was 40cm/CSF. Lumbar punctures were performed at least weekly in HIV-infected patients for the first 4 weeks and at 10 weeks, and more frequently if the pressure was raised or the patient had symptoms of raised intracranial pressure (headache, vomiting). Frequency of lumbar puncture in HIV uninfected patients was at the physician's discretion guided by symptoms/signs suggestive of raised intracranial pressure.

2.5.10 Follow up

All patients were followed up for 6 months.

2.6 Microbiological methods

2.6.1 Identification

Isolation and identification of *Cryptococcus species* was made using standard microbiological methods. Initially India ink stain of CSF was used to demonstrate the presence of budding encapsulated yeasts on India ink stain of cerebrospinal fluid (CSF). The CSF pellet was cultured at 35°C on chocolate and blood agar, brain-heart infusion broth, and at 30°C and 35°C on Sabouraud's agar (Oxoid, Basingstoke, UK). Identification was confirmed through demonstration of the growth of characteristic colonies on Sabouraud's and bird seed agar, demonstration of urease production (Christensen's agar) and API 32C sugar assimilation tests (BioMerieux SA, Marcy l'Etoile, France). Isolates were stored at -30°C on Microbank plastic beads (Pro-Lab Diagnostics, Neston, Cheshire, UK). Strains were revived for speciation on Sabouraud's agar and incubated at 30C and 37*C. C. gattii* was identified by biotyping with Canavanine Glycine Bromothymol Blue (CGB) agar, and isolates were further divided into eight molecular groups using Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the *URA5* (orotidine monophosphate pyrophosphorylase) gene [35]. Control strains were provided by Dr Wieland Meyer, Westmead Millennium Institute for Medical Research, Sydney, Australia.

2.6.2 Cryptococcal Antigen Testing

Antigen testing was performed on patient serum (qualitative) and cerebrospinal fluid (quantitative) using the Cryptococcal Antigen Latex Test R30851501 (Remel, Lenexa,

KS, USA) as per the manufacturer's instructions. A positive result was determined using the internal low positive control, equating to a level of antigen of 12ng/ml.

2.6.3 Determination of Cryptococcal Load in CSF

Cryptococcal fungal burden was estimated in 2 ways. All patients had *Cryptococcus* antigen titres measured in CSF at presentation. In addition, all HIV patients had an assessment of fungal burden through measurement of the number of colony forming units (CFUs) of *Cryptococcus* per ml of cerebrospinal fluid using a serial dilution technique. Briefly, 100uL of fresh CSF was serially diluted 4 times in 0.9ml of sterile water. CSF and subsequent dilutions were vortexed for 5 seconds before removal and after addition of each 100uL aliquot. After vortexing each dilution, 100uL of neat CSF /diluent was inoculated in 4 – 5uL aliquots onto the surface of a Sabouraud's plate, in duplicate. Plates were incubated at 30C for 1 week and CFUs were counted daily. The mean number of CFUs was calculated using the duplicate inoculations from the plates that had between 20 and 130 Colony Forming Units. All patient CSF samples were processed within 4 hours.

2.6.4 Antifungal Sensitivity Testing

I used the Sensititre® YeastOne® (Trek Diagnostic Systems, East Grinstead, UK) microbroth susceptibility testing plates to determine the MICs of 8 antifungal drugs against all the *Cryptococcus* species in the OUCRU strain collection. The Sensititre® YeastOne® system is a broth microdilution method that uses a 96 well plate format. A colorimetric indicator, Alamar Blue, denotes the presence of growth within a well by

changing from blue to pink (Figure 2.4). Alamar Blue is water soluble and non-toxic to cells. The colour change is a result of reduction by a number of intracellular molecules including NADPH, NADH and cytochromes[244]. Plates are read with the naked eye under normal laboratory lighting using a reading mirror. The antifungals tested were: amphotericin B, flucytosine, fluconazole, ketoconazole, itraconazole, posaconazole, voriconazole and caspofungin. *Cryptococcus* species are inherently resistant to caspofungin.

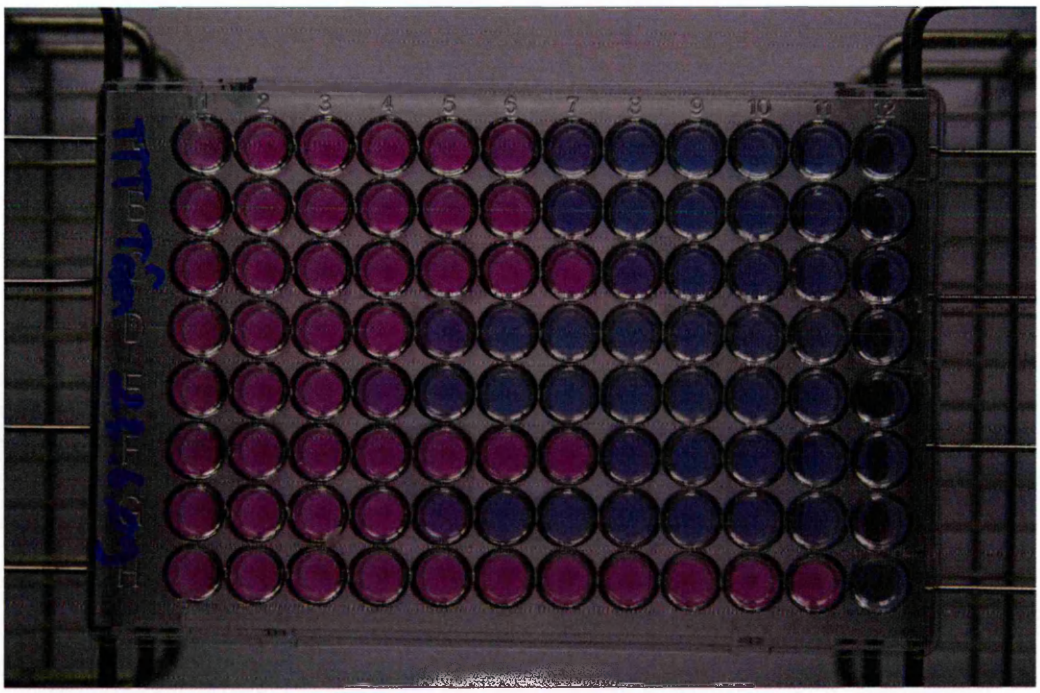


Figure 2.4 Sensititre YeastOne antifungal susceptibility plate. Growth results in colour change from blue to pink.

MICs were determined in accordance with the manufacturer's instructions, with one adaptation – I adjusted the inoculum to 1.0 McFarland rather than 0.5 McFarland, since this more consistently resulted in the recommended final organism density of 1.5 – 8X 10^3 colony forming units (CFUs) per ml. Briefly, inocula were made from a 24 hour old growth of pure isolate added to sterile water. 20uL were transferred to 11ml YeastOne®

culture medium (broth). Suspensions were vortexed for 15 seconds during adjustment and prior to addition to broth. The broth was transferred to a sterile seed trough and an 8 channel pipette was used to inoculate the 96 well plate. 10ul of suspension was removed from the positive control well and plated onto a Sabouraud's agar plate to check purity and final pathogen density. A correct final pathogen density of $1.5 - 8x10^3$ CFUs per ml would result in between 15 – 80 CFUs on the purity plate. Plates were covered with an adhesive seal and incubated at 35C, stacked no more than 3 high as per the manufacturer's instructions. Since the inoculum preparation step and the subsequent plate inoculation should take no more than 15 minutes each, no more than 4 isolates were processed at one time, the duration of the process being measured with a stopwatch. The MICs were estimated at 48 and 72 hours following inoculation. MICs were not estimated unless there was colour change in the positive control well, and the organism density was in the correct range. The MICs were identified as the lowest drug concentration preventing the development of red or purple broth discolouration (growth) – i.e. the first blue well. The ranges of drug concentration tested are detailed in table 2.1 below. All isolates were obtained prior to the instigation of treatment.

Quality control was performed using *Issatchenkia orientalis* (formerly *Candida krusei*) ATCC 6258 and *Candida parapsilosis* ATCC 22019, and was performed with every 7 isolates for the first 49 isolates, and then every 47 isolates.

All results were entered onto a spreadsheet (Microsoft Excel 2003, Microsoft Corporation, Seattle, USA).

Table 2.1

Concentration ranges of the 8 antifungal drugs in the Sensititre® YeastOne® test plate.

Antifungal Agent	Dilution range (ug/ml)
Posaconazole	0.008 - 8
Amphotericin B	0.008 – 16
Fluconazole	0.125 – 256
Itraconazole	0.008 – 16
Ketoconazole	0.008 – 16
5-Flucytosine	0.03 – 64
Voriconazole	0.008 – 16
Caspofungin	0.008 - 16

2.6.5 Control strains

Control strains used for antifungal sensitivity testing were *Issatchenkia orientalis* ATCC 6258 and *Candida parapsilosis* ATCC22019.

Control strains for typing were provided by Dr Wieland Meyer, Westmead Millennium Institute for Medical Research, Sydney, Australia (Table 2.2).

Table 2.2

***Cryptococcus* control strains for molecular typing**

Species	Code	*URA5* RFLP Group [35]	Serotype
Cryptococcus neoformans var *grubii*	WM148	VNI	A
Cryptococcus neoformans var *grubii*	WM626	VNII	A
Cryptococcus neoformans hybrid	WM628	VNIII	AD
Cryptococcus neoformans var *neoformans*	WM629	VNIV	D
Cryptococcus neoformans var *gattii*	WM179	VGI	B
Cryptococcus neoformans var *gattii*	WM178	VGII	B
Cryptococcus neoformans var *gattii*	WM175	VGIII	B
Cryptococcus neoformans var *gattii*	WM779	VGIV	C

2.7 Molecular Methods

2.7.1 DNA Extraction

Whole genomic DNA was extracted according to the method of Wen et al[245]. Briefly, isolates stored at -30C (Microbank plastic beads, Pro-Lab Diagnostics, Neston, Cheshire, UK) were revived on Sabouraud's agar and incubated at 30C for 3 days. Single colonies were spread for confluent growth on Sabouraud's plates and incubated at 30C for 24 hours. All cell growth on the plate (approximately 0.5g wet weight) was used for DNA extraction. Cells were resuspended in sodium citrate sorbitol buffer (SCS) containing 20mM sodium citrate and 1M D-sorbitol (Sigma-Aldrich), pH adjusted to 5.8 and centrifuged at 13000g for 5 minutes. This process was repeated, and the pellet was

then resuspended and incubated in freshly made protoplasting solution (10 mg lysing enzymes from *Trichoderma harzianum*, Sigma in 1 ml SCS solution) at 37C for 1 hour. The solution was centrifuged at 13000g for 5 minutes and the pellet resuspended in 1 ml of lysing solution (8% DTAB (Dodecyltrimethylammoniumbromide Sigma D-8638), 1.5 M NaCl, 100 mM Tris (pH 8.6), 50 mM EDTA (pH 8)). The suspension was separated into 2 eppendorfs, gently shaken and incubated at 68C for 30-60 minutes. After cooling to room temperature 0.5ml of fridge-cold chloroform was added to each tube, mixed by inversion and centrifuged at 4C at 13000g for 10 minutes. The supernatant was added to 0.5 x volume of 5% CTAB solution (cetyltrimethylammoniumbromide, Sigma Aldrich, with 0.4 M Nacl) and 2 x volume of ELGA water. After gentle mixing this was centrifuged (13000g for 2 minutes) at 4C. The supernatant was discarded and the DNA-CTAB pellet resuspended in 1.2M NaCl. 1 ml of ice cold 100% ethanol was added and mixed by inversion before re-centrifuging tube (12 000 rpm for 10 min at 4 C). The pellet was rinsed in 1ml of ice-cold 70% ethanol and re-centrifuged (12 000 rpm for 5 min at 4C). The supernatant was discarded and the pellet dried, after which it was resuspended in 100uL of TE buffer and left to dissolve overnight. One uL of 100mg/ml RNAse was added, and the presence of DNA was confirmed through electrophoresis on a 1% agarose gel. DNA was quantified using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

2.7.2 Restriction Fragment Length Polymorphism (RFLP) Analysis

RFLP of the orotidine monophosphate pyrophosphorylase (*URA5*) gene was carried out according to the methods of Meyer et al [35]. PCR of the *URA5* gene was conducted in a

final volume of 50uL. Each reaction contained 50 ng of DNA, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2; Applied Biosystems, Foster City, CA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics GmbH), 3 mM magnesium acetate, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and 50 ng of each primer URA5 (5'ATGTCCTCCCAAGCCCTCGACTCCG 3') and SJ01 (5' TTAAGACCTCTGAACACCGTACTC 3'). PCR was performed for 35 cycles in a PerkinElmer 480 Thermal Cycler (PerkinElmer Inc, Massachusetts, USA) at 94°C for 2-min initial denaturation, 45 s of denaturation at 94°C, 1 min annealing at 61°C, and 2-min extension at 72°C, followed by a final extension cycle for 10 min at 72°*C*. Successful amplification was confirmed through demonstration of a 779bp product on electrophoresis (10uL of product, 1% agarose gel, 110V, 45 minutes).
20 μL of PCR products were double digested with 2uL Sau96I (10 U/uL) and 2uL HhaI (40 U/μl), with 5uL of NE buffer 4 and 0.5uL of bovine serum albumin, made up to a final volume of 50uL with ELGA water. The reaction mix was incubated at 37C for 3 hours at and 20ul of final product was separated by 3% agarose gel electrophoresis at 100 V for 3 h. RFLP patterns were assigned visually by comparing them with the patterns obtained from the standard strains (VNI-VNIV and VGI-VGIV) provided by Associate Professor Wieland Meyer, University of Westmead, Sydney.

2.7.3 Amplified Fragment Length Polymorphism Analysis (AFLP)

AFLP was based upon the Carter lab protocol, a modification of the method described by Halliday et al [246].

2.7.3.1 Restriction digest

Each reaction mix contained 1.3uL of EcoR1 (Helena Biosciences Europe, Gateshead, UK, 10U/uL) and 0.7uL Mse1 (Helena Biosciences Europe, Gateshead, UK, 10U/uL), 20uL of genomic DNA (20 – 60ng), 4uL of One-phor-all buffer (Amersham Biosciences, GE Healthcare Life Sciences, Little Chalfont, UK) and ELGA water to a final reaction mix of 40uL. To check adequate digestion, 10uL of reaction mix was removed to a fresh tube and 1uL of lambda DNA (Promega UK Ltd, Southampton, UK, 50ng/uL). Samples and digestion checks were incubated at 37C for at least 3 hours. The lambda digest was run on a 1% agarose gel to check there were no undigested 5kb lambda DNA fragments.

2.7.3.2 Adapter Ligation

Double stranded oligonucleotide adapters were prepared by boiling high concentrations (100uM) of complementary single stranded oligonucleotides (EA1 and EA2 or MA1 and MA2) for 2 minutes. Adapter sequences are illustrated in table 2.3.

7.5uL of ligation mix (0.75µl 10x One-phor-all buffer, 0.75µl ATP (Invitrogen, 10mM), 0.1875µl T4 ligase (Helena Biosciences Europe, Gateshead, UK, 4 Weiss units/µl), 0.75µl EcoR1 adapters (5µM), 0.75µl Mse1 adapters (50µM), and 4.3125µl ELGA water were added to each 30uL restriction digest mix. Ligation mix was incubated at room temperature (25C) overnight.

2.7.3.3 PCR amplification

Preselective and selective amplification primers are detailed in Table 2.3. The AFLP preamplification and selective amplification reactions were performed in a final volume of 25 µl. The preamplification reaction mixture contained 1 X PCR buffer, 5 nM concentrations of each deoxyribonucleotide triphosphate, 2 µM concentrations of the appropriate EcoRI and MseI preamplification primer pairs or selective primers, 5 U of Taq DNA polymerase (Amersham Biosciences, GE Healthcare Life Sciences, Little Chalfont, UK), and 1 µl of digestion ligation product.. The selective amplification mixture contained 0.5µM final concentrations of each primer. The selective primer pairs used in this study were: EcoRI-GT–MseI-GT, and EcoRI-AC– MseI-G. The selective EcoRI primer was labelled at the 5' end with 6-carboxyfluorescein (6 FAM). The preamplification reaction conditions were 94°C for 2 minutes followed by 20 cycles of 94°C for 20 s, 56°C for 30s, and 72°C for 2 min. The selective amplification conditions were 94°C for 2 minutes followed by 10 cycles of 94°C for 20 s, 66°C for 30 s, decreasing 1°C every cycle, and 72°C for 120 s, followed by 20 cycles consisting of 94°C for 20 s, 56°C for 30 s, and 72°C for 120 s. All amplification reactions were performed in a BioRad C1000 thermal cycler (BioRad Laboratories Ltd, Hemel Hempstead, UK).

Table 2.3

Oligonucleotides – Adapters and Primers

Adapter	Oligo name	Sequence
EcoR1	EA1	5'-CTCGTAGACTGCGTACC-3'-
	EA2	3'-CATCTGACGCATGGTTAA-5'-
Mse1	MA1	5'-TACTCAGGACTCAT-3'
	MA2	3'-GAGTCCTGAGTAGCAG-5'

Preselective Primer Pairs

EcoR1-A	5'-GACTGCGTACCAATTCA-3'
Mse1-G	5'-GATGAGTCCTGAGTAAG-3'
EcoR1-G	5'-GACTGCGTACCAATTCG-3'
Mse1-G	5'-GATGAGTCCTGAGTAAG-3'

Selective Primer Pairs

EcoR1-AC	5'-FAM6-GACTGCGTACCAATTCAC-3'
Mse1-G	5'-GATGAGTCCTGAGTAAG-3'
EcoR1-GT	5'-FAM6-GACTGCGTACCAATTCGT-3'
Mse1-GT	5'-GATGAGTCCTGAGTAAGT-3'

2.7.3.4 AFLP Fragment Detection

Fragment analysis was performed using an Applied Biosystems 3130X 16 capillary (50cm) sequencer with Genemapper version 4.0 software (Applied Biosystems Inc, CA, USA). Amplification product was diluted as necessary, mixed with LIZ 500 size standard and HiDi Formamide (AB Biosystems Inc, CA USA), vortexed, heated to 95C for 3 minutes and then snap-cooled on ice before loading and using the default AFLP electrophoresis settings as recommended by the manufacturer.

2.7.3.5 Analysis

Raw file data was imported into Bionumerics Version 5.1 (Applied Maths, Belgium) for analysis. 144 files were imported simultaneously and normalised. Further details are given in the relevant chapter.

2.8 Statistical Methods

Statistical analysis was performed with R version 2.9.0 (R Foundation for Statistical Computing, Vienna, Austria) [247]. Statistical methods will be described in each individual chapter.

Chapter 3

The Clinical Phenotype of Cryptococcal Meningitis and the Impact of HIV.

3.1 Introduction

HIV undoubtedly increases the risk of developing cryptococcal meningitis - the HIV epidemic, with over 40 000 000 people currently affected worldwide, has produced an epidemic of cryptococcal meningitis in its wake. There are estimated to be up to 1.5 million cases per year world wide, with up to 1 125 000 deaths; in sub-Saharan Africa cryptococcal meningitis now probably outstrips even tuberculosis as a cause of death [1]. HIV-associated disease justifiably dominates research efforts and subsequently guides developments in treatment. However, while HIV-associated disease represents the vast majority of cases, disease is not limited to this group. The first culture-proven case of cryptococcal meningitis was described well before the HIV pandemic in 1912 by Rusk and Farnell, and there has been speculation that a case of chronic meningitis described in 1860 was due to *Cryptococcus neoformans* [248]. Thus disease in the HIV uninfected is well recognised. However, it remains rare, and the differences in clinical phenotype between HIV infected and uninfected patients are not clear. Differences in clinical phenotype, presumably a result of differences in disease pathogenesis, may have implications for effective treatment, which might need to be tailored to the immune status of the patient. Since disease in the HIV uninfected often occurs in patients with other underlying immunosuppressive disease, or is due to infection with *C. gattii*, it is difficult to ascribe differences in clinical phenotype to HIV alone. At the Hospital for

Tropical Diseases, cryptococcal meningitis in the HIV uninfected is infrequently accompanied by other underlying disease. Moreover, my work suggests that most infections are due to *Cryptococcus neoformans* var *grubii*, the same species responsible for disease in the HIV infected patients. This affords the opportunity to define the impact of HIV on clinical phenotype in cryptococcal meningitis. In this chapter I review the literature regarding the clinical phenotype of cryptococcal meningitis and examine the effect of HIV on clinical phenotype at the Hospital for Tropical Disease. In addition, I assess the validity of previously described prognostic factors in cryptococcal meningitis.

3.2 Aims

1. To compare the clinical phenotypes of cryptococcal meningitis in the published literature according to HIV serostatus.
2. To determine the impact of HIV infection on the presentation of cryptococcal meningitis in southern Viet Nam.
3. To assess the reliability of previously defined prognostic factors in cryptococcal meningitis.

3.3 Methods

3.3.1 Literature review of clinical phenotypes and prognostic factors

Publications describing the clinical phenotype of cryptococcal meningitis were identified from the PUBMED database using the following search terms:

1. <*Cryptococcus neoformans*> with the LIMITS <clinical trials> and <humans> and <English Language>.
2. Using the MeSH terms: "Meningitis, Cryptococcal"[Mesh] AND "Prognosis"[Mesh] AND ("Clinical Trial "[Publication Type] OR "Descriptive Studies"[Mesh]).

Studies that detailed presenting findings were tabulated and the numbers of patients reported with and without particular symptoms were extracted, according to HIV status. Findings from different studies were synthesized using standard meta-analytic techniques. In particular, I visualized effects using forest plots and assessed between-study heterogeneity using the I^2 statistic [249, 250]. The Chi squared test was used to compare the proportions of patients with each symptom or sign by HIV serostatus. Analyses were done with the statistical programme R version 2.9.0 (R Foundation for Statistical Computing, Vienna, Austria) [247] and the contributed R-package meta version 1.1-5. Presence of the following symptoms/signs was analysed: fever, headache, malaise, neck stiffness, meningeal signs, nausea/vomiting, visual impairment, focal neurological signs, fits and confusion. If studies reported neck stiffness then this was analysed both separately and carried forward to the meningeal sign group.

3.3.2 Prospective comparison of clinical phenotype of cryptococcal meningitis in Vietnamese patients according to HIV infection status.

3.3.2.1 Patients

All patients at the Hospital for Tropical Diseases, Ho Chi Minh City, with suspected or definite central nervous system infections are referred to the Viet-Anh (Clinical Research) Ward where they are assessed and cared for by a dedicated Clinical Research team of doctors, nurses and allied professionals. All patients in this analysis were recruited to one of 2 studies – the BMD study (HIV uninfected patients) or the BK study (HIV infected patients), described in Chapter 2. Both studies were based solely in the Hospital for Tropical Diseases. The studies had ethical approval from the Hospital for Tropical Diseases and either the Oxford University Tropical Research Ethics Committee (BMD study) or Liverpool School of Tropical Medicine Research Ethics Committee (BK Study).

The clinical assessment, investigations, treatment and microbiological methods are detailed in Chapter 2.

3.3.2.2 Statistical Methods

Baseline variables were compared between HIV infected and uninfected patients using Fisher's exact test for categorical data and the Wilcoxon rank sum test for continuous data. All analyses were performed with R version 2.9.0 (R Foundation for Statistical Computing, Vienna, Austria [247]).

3.3.3 Assessment of Prognostic Factors

The effect of previously published predictors for survival in cryptococcal meningitis patients was determined in this cohort of Vietnamese patients.

Previously published predictors were identified through a literature search of PUBMED (http://www.ncbi.nlm.nih.gov/sites/entrez) using the terms <*Cryptococcus*> <meningitis> <outcome> <prognostic factors> and using the limits <human> and <clinical trial>. The search was repeated using the MeSH terms "Meningitis, Cryptococcal"[Mesh] AND "Prognosis"[Mesh] AND ("Clinical Trial "[Publication Type] OR "Retrospective Studies"[Mesh]). Both survival within 10 weeks and survival within 6 months following presentation were studied. Patients who did not die in the respective time interval were censored at the end of the interval or at the time of loss to follow-up, respectively. The Cox proportional hazards regression model was used to study the association between covariates and outcome. In a first step, the univariate association between candidate predictors and outcome was studied. Continuous covariates with a severely right-skewed distribution were log10-transformed prior to the analysis (after adding 1 to the original value to deal with zero measurements in the case they occurred). Cut-offs for continuous covariates were only studied if they had been previously published as in general dichotomizing may create rather than avoid problems, notably a considerable loss of power and residual confounding. In addition, the use of a data-derived 'optimal' cutpoint can lead to serious bias [251, 252]. In a second step, I developed a multivariate Cox regression model as follows: All covariates which achieved a p-value of <0.20 in the univariate analysis and were missing in less than 20% of patients were included as candidates in the multiple regression model. Backward

model selection was performed according to Akaike's information criterion (AIC) to select a more parsimonious model. Finally, the stability of the backwards model selection was determined by investigating the variability of the model selection in 100 bootstrap resamples of the original dataset [253]. Of note, this investigation may overestimate the true stability as it only validated the backwards selection but not the initial screening of the covariates for inclusion in the multivariate model.

No imputation of missing data was performed and effects were displayed as hazard ratios with the two-sided 95% confidence interval.

3.4 Results

3.4.1 Literature review of the clinical phenotype of cryptococcal meningitis

The literature search identified 140 studies. Of these, 24 studies were found where the clinical presentation of cryptococcal meningitis was described by HIV serostatus and the raw data including outcome were extractable [57, 61, 62, 76, 99-107, 127, 144, 157, 254-261]. While some papers presented large or historically significant series, it was not always possible to extract the raw data for patients with meningoencephalitis (other cryptococcal disease was included) or to compare by HIV serostatus [70, 154, 156, 262]. In total, data were available for 2204 patients, 1541 HIV infected and 663 HIV uninfected. The raw numbers assessed and reporting each symptom and sign, prevalences, odds ratios and P values for differences by serostatus are shown in table 3.1 below. While in theory it would have been possible to compare the results of laboratory investigations between studies using a t-test, in practice the mean values and standard deviations of laboratory parameters were rarely presented in the papers and thus

concatenation and comparison of results was not possible. The *Cryptococcus* species causing disease is not determined in most patients; therefore, I have not attempted to distinguish clinical syndrome by infecting species.

Table 3.1

Concatenation of published rates of symptoms and signs in 2204 patients with cryptococcal meningitis by HIV serostatus.

	N/Total		% (Range)				
Total Patients reported	**1541**	**663**					
Symptom/Sign	**HIV Infected**	**HIV Uninfected**	**HIV Infected**	**HIV Uninfected**	**OR**	**95% CI**	**P**
Fever	799/1352	285/466	59.1(39 – 86)	61.2 (33 – 85)	0.92	0.73 – 1.14	0.44
Headache	1142/1500	379/517	76.1 (46 - 100)	73.3 (45 – 91)	1.16	0.92 – 1.47	0.21
Neck stiffness	372/752	56/84	49.5 (16 - 93)	66.7 (67 - 67)	0.49	0.29 – 0.80	0.003
Meningeal Signs	565/1305	160/335	46.3 (19 - 93)	47.8 (25 - 69)	0.84	0.65 – 1.07	0.16
Photophobia	22/177	3/63	12.5 (5.0 - 18)	4.8 (4.8)	2.83	0.81 – 15.3	0.10
Confusion/impaired conscious level	433/1403	270/605	30.9 (6.0 - 58)	44.6 (26 - 71)	0.55	0.45 – 0.68	<0.001
Focal neurological signs	78/334	108/283	23.4 (6.0 - 50)	38.2 (27 - 50)	0.49	0.34 – 0.71	<0.001
Fits	45/520	65/494	9.0 (1.5 - 18)	13.2 (5.0 - 24)	0.63	0.41 – 0.95	0.03
Visual impairment	75/554	71/314	13.5 (9.5 - 15)	22.6 (16 - 34)	0.54	0.54 – 0.78	<0.001
Nausea or Vomiting	262/583	98/157	45.0 (31 - 70)	62.4 (49 - 72)	0.49	0.34 – 0.72	<0.001
Respiratory Symptoms	165/754	33/157	21.9 (17 - 38)	21.0 (21)	1.05	0.68 – 1.66	0.92

The range refers to the maximum and minimum percentages of patients reported to have the symptom or sign in individual studies. From this crude analysis, it appears that there are some significant differences in the clinical phenotypes of cryptococcal meningitis according to HIV serostatus. The prevalence of fever, headache, photophobia and respiratory symptoms were no different by HIV serostatus, but neck stiffness, confusion, focal neurological signs, fits, visual impairment and nausea or vomiting were all statistically significantly more common in the HIV uninfected group. There was no difference in prevalence of the collective symptom 'meningeal signs', although this was not always clearly defined in the reports. I tested the heterogeneity of the patient populations presented in the publications using the I^2 statistic. The I^2 statistic describes the percentage of variation across studies that is due to heterogeneity rather than chance [249, 250]. It is based on the traditional measure of variance, the Cochran Q statistic [263]. Unlike Q it does not inherently depend upon the number of studies considered. Substantial heterogeneity exists when I^2 exceeds 50%. The analyses revealed that both the HIV infected and HIV uninfected patient populations were highly heterogeneous – i.e. there were large variations in the reported prevalence of a particular symptom or sign within a superficially similar group (Table 3.2). The heterogeneity between study populations is further illustrated by the Forest plots for the symptom/sign prevalence by HIV serostatus (Figures 3.1 – 3.7).

Table 3.2

Heterogeneity testing of symptom/sign prevalences in cryptococcal meningitis in published series by HIV serostatus

	HIV infected		HIV uninfected	
Symptom	I^2 value	95% CI	I^2 value	95% CI
Fever	92.7	89.3 - 95	86.3	72.4 – 93.2
Headache	96.2	94.9 – 97.2	86.1	74.6 – 92.4
Neck Stiffness	96.3	94.4 – 97.5	0.0	0.0
Meningeal Symptoms	97.3	96.4 – 98.0	95.5	91.3 – 97.6
Confusion	94.5	92.2 – 96.2	2.4	0 – 65.6
Focal deficit	93.9	88.5 – 96.7	0	0 - 43
Fits	70.8	32.1 – 87.5	81.0	59.3 – 91.2
Visual Impairment	29.2	0 – 92.6	81.1	41.1 - 94
Respiratory Symptoms	76.6	43.2 – 90.4	NA*	NA

* Only 1 study reported presence or absence of respiratory symptoms for HIV uninfected patients.

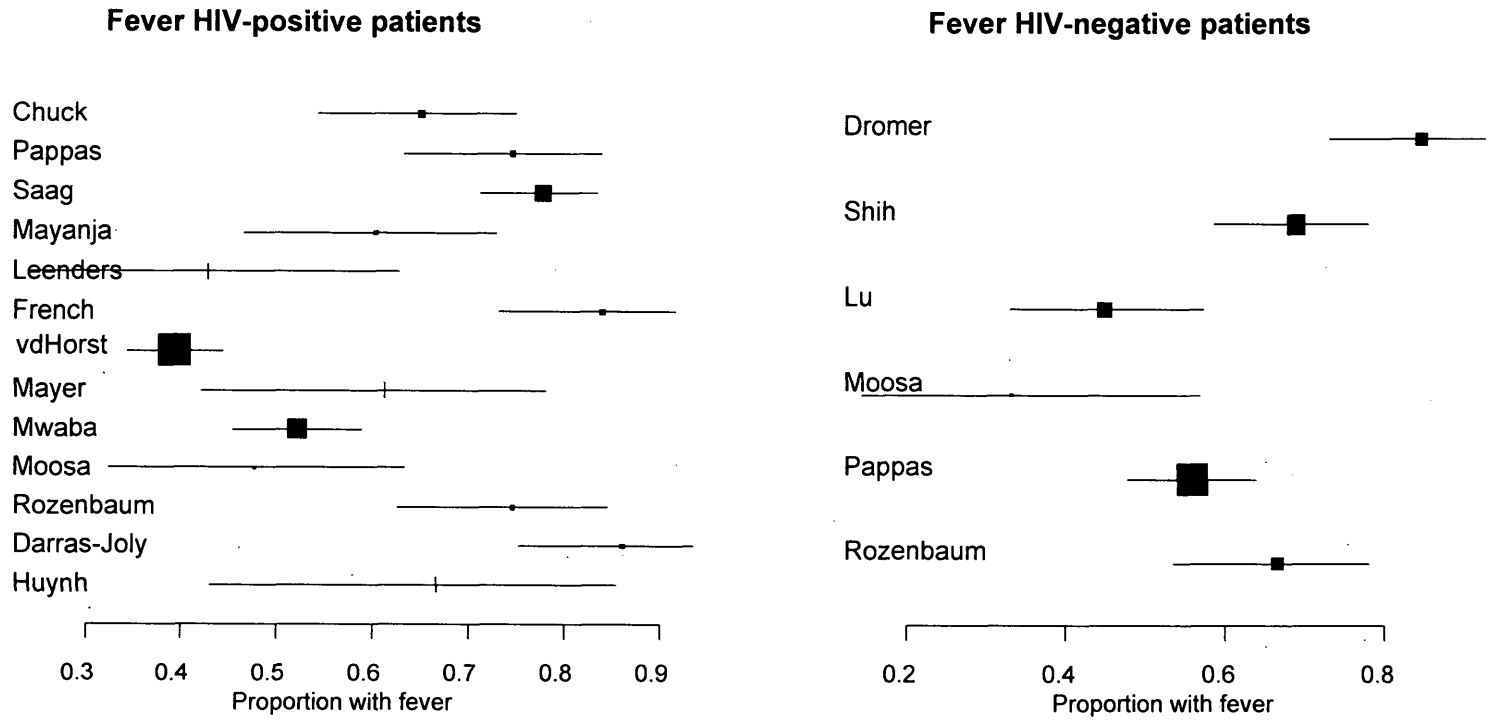
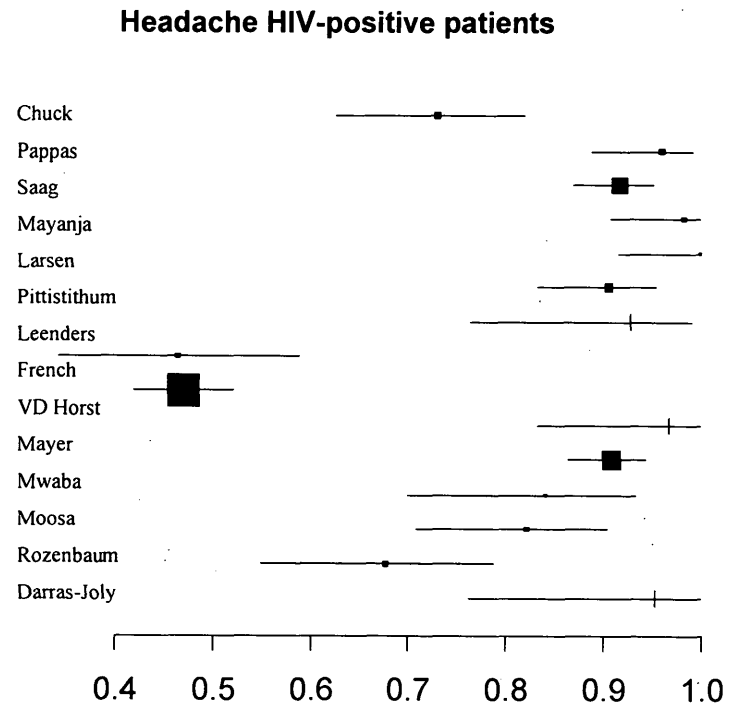


Figure 3.1. Forest plots comparing proportion of patients with cryptococcal meningitis in published series with fever according to HIV serostatus (names refer to first author [57, 61, 62, 76, 96-104, 123, 136, 148, 244-251]).



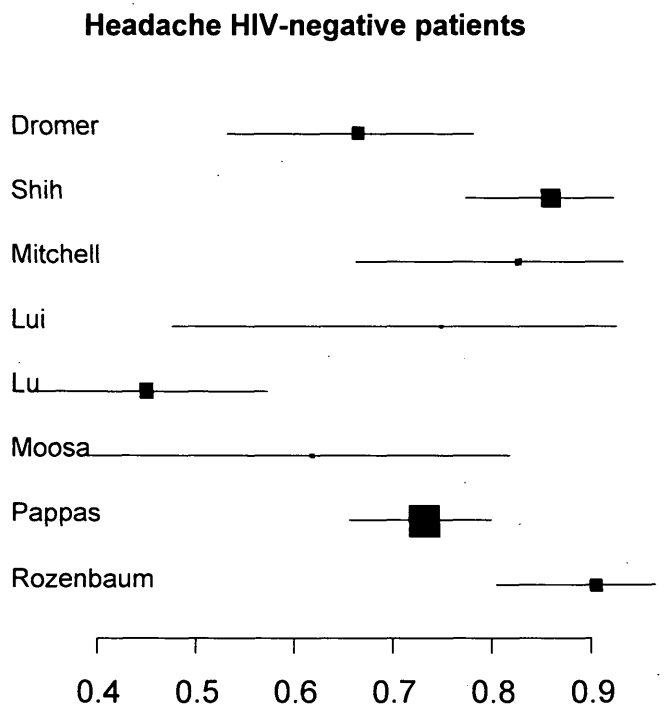


Figure 3.2. Forest plots comparing proportion of patients with cryptococcal meningitis in published series with headache according to HIV serostatus (names refer to first author [57, 61, 62, 76, 96-104, 123, 136, 148, 244-251]).

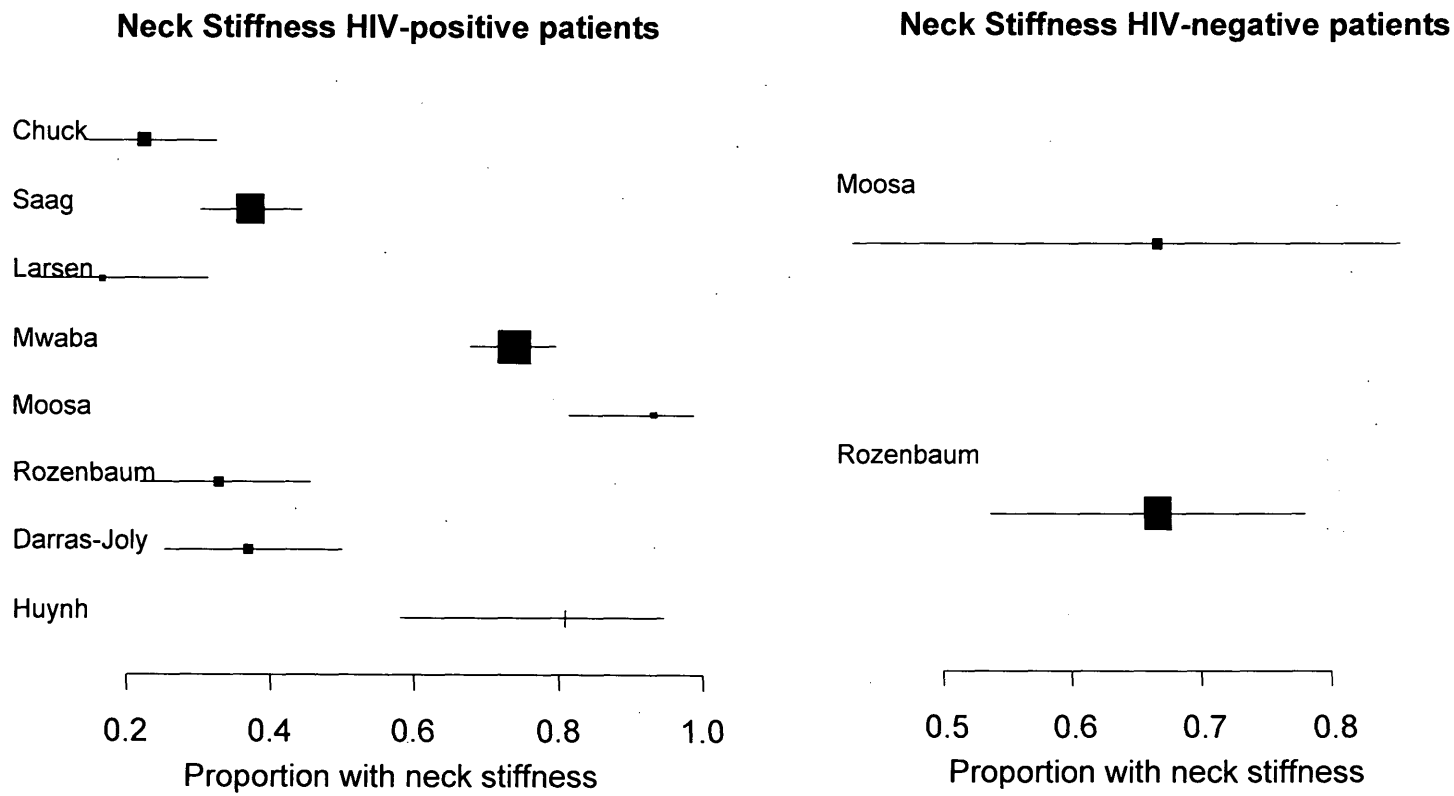


Figure 3.3. Forest plots comparing proportion of patients with cryptococcal meningitis in published series with neck stiffness according to HIV serostatus (names refer to first author [57, 61, 62, 76, 96-104, 123, 136, 148, 244-251]).

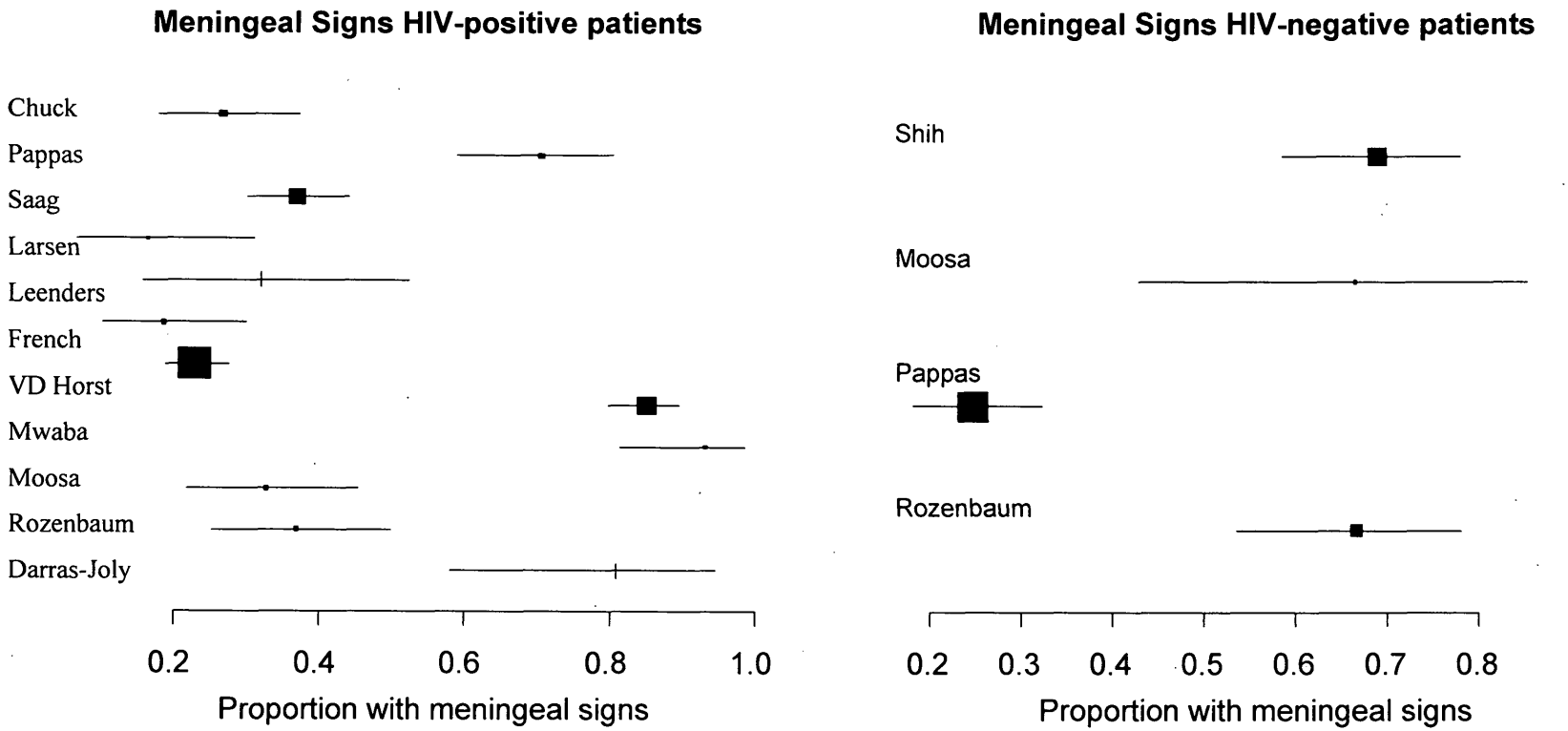


Figure 3.4. Forest plots comparing proportion of patients with cryptococcal meningitis in published series with meningeal signs according to HIV serostatus (names refer to first author [57, 61, 62, 76, 96-104, 123, 136, 148, 244-251]).

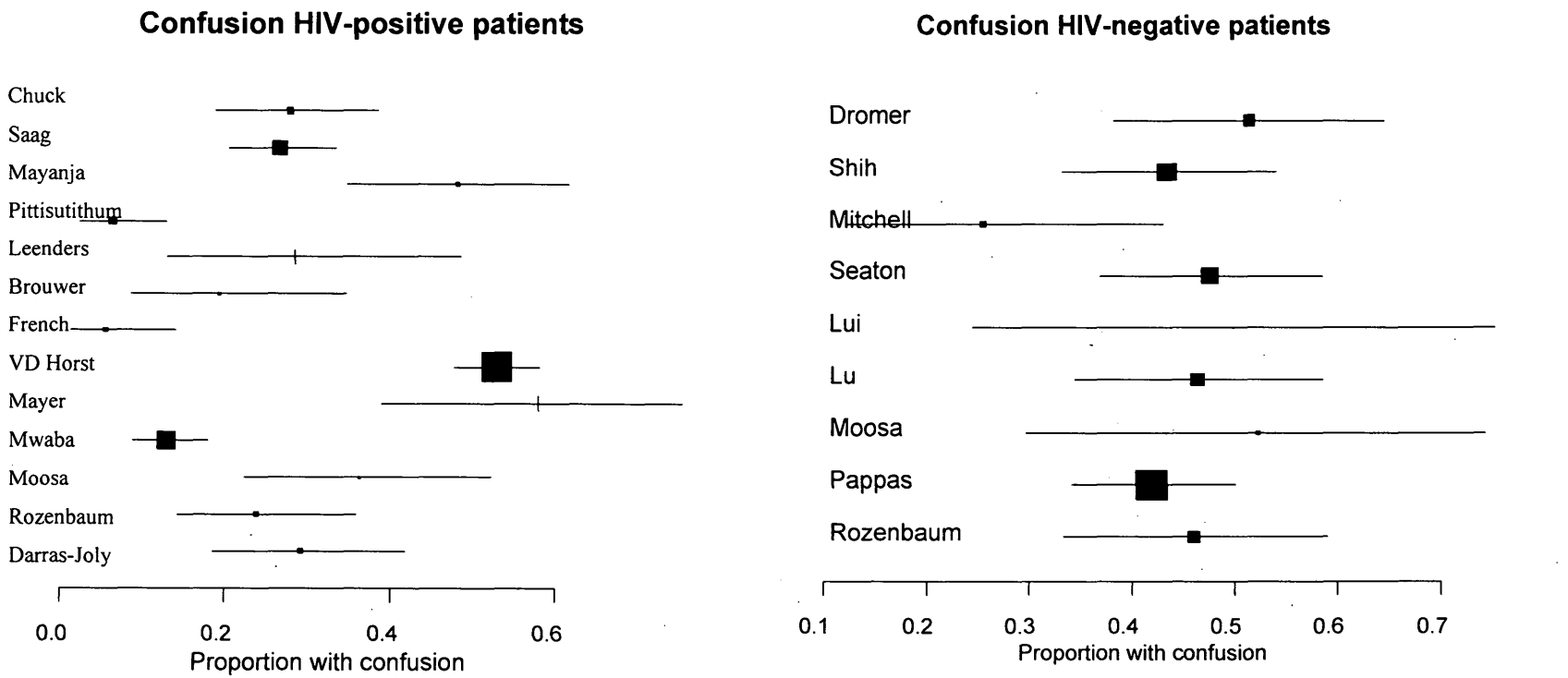


Figure 3.5. Forest plots comparing proportion of patients with cryptococcal meningitis in published series with confusion according to HIV serostatus (names refer to first author [57, 61, 62, 76, 96-104, 123, 136, 148, 244-251]).

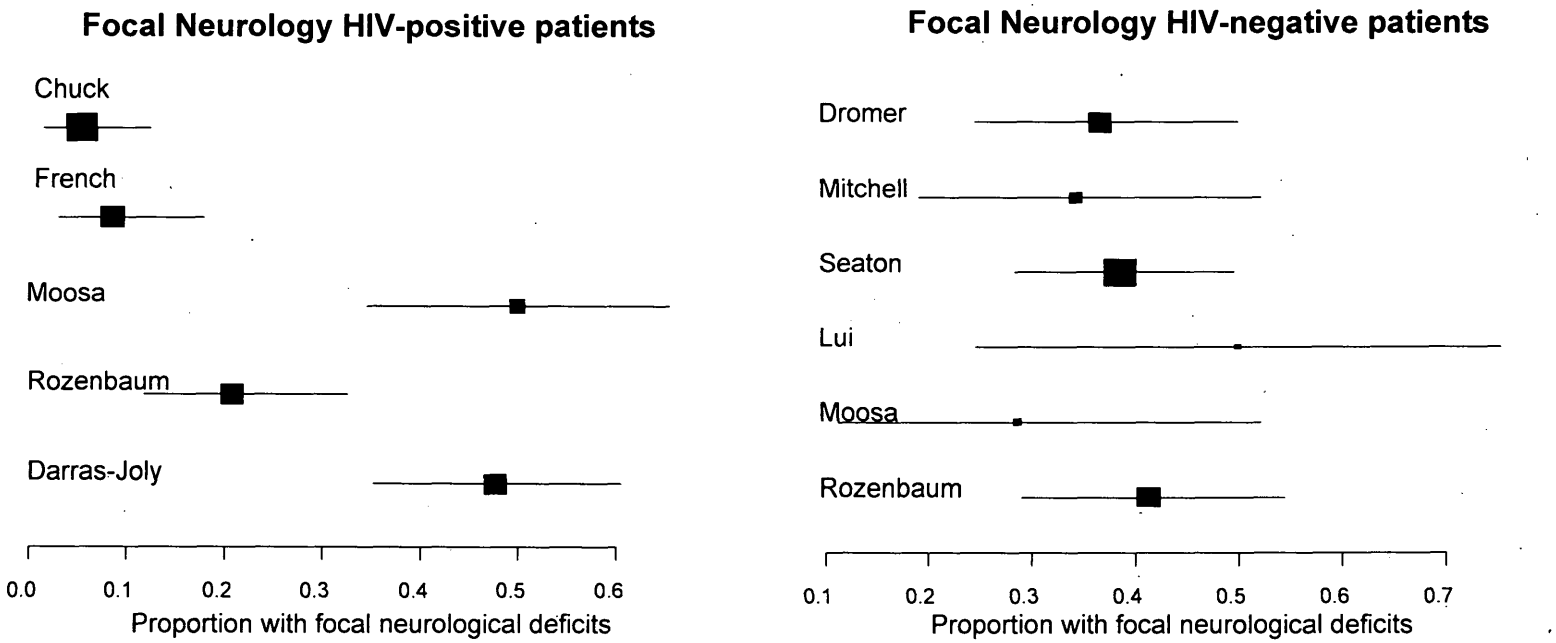
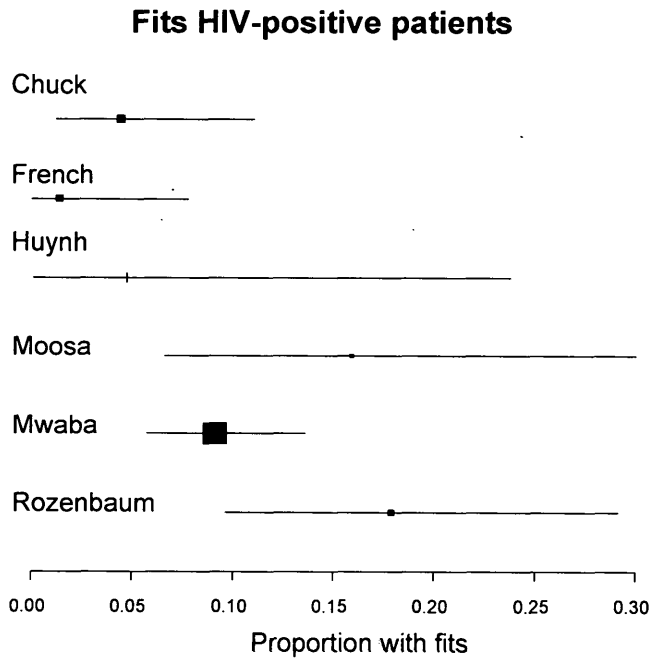


Figure 3.6. Forest plots comparing proportion of patients with cryptococcal meningitis in published series with focal neurology according to HIV serostatus (names refer to first author [57, 61, 62, 76, 96-104, 123, 136, 148, 244-251]).



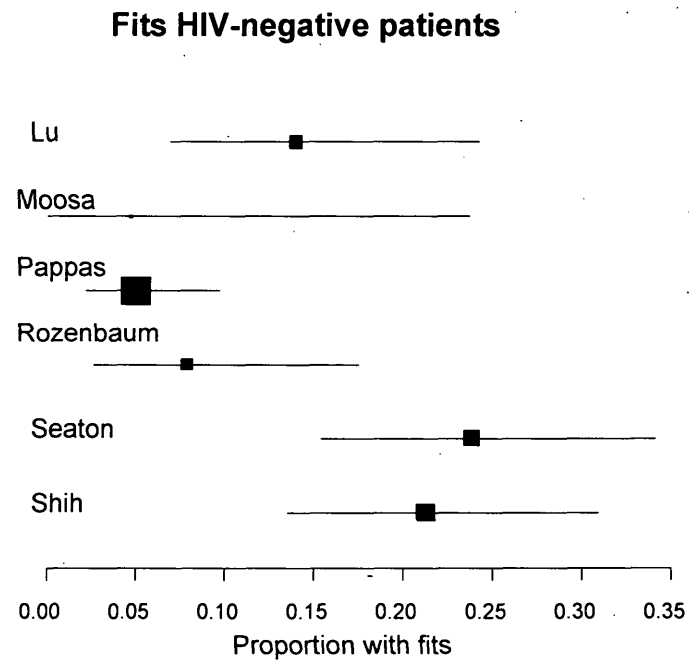


Figure 3.7. Forest plots comparing proportion of patients with cryptococcal meningitis in published series with fits according to HIV serostatus (names refer to first author [57, 61, 62, 76, 96-104, 123, 136, 148, 244-251]).

3.4.2 Clinical Phenotype in southern Viet Nam

57 HIV uninfected patients and 67 HIV positive patients were recruited during the study period. Of the 57 HIV uninfected patients, eleven (19.3%) had potentially immunosuppressive pre-existing health problems. Two patients had cirrhosis, one had both cirrhosis and renal impairment and one had diabetes mellitus. Seven patients had taken more than three months of corticosteroid therapy (2 for nephrotic syndrome, 2 for Evan's syndrome, 2 for chronic arthritides and 1 for systemic lupus erythematosus).

3.4.2.1 Demography and symptoms

Demographic findings and symptoms on presentation are presented in Table 3.3 below: HIV infected patients were younger (median age 26 years versus 37 years, p=0.002) and more likely to be male (88.1% versus 54.4%, P<0.001). The median duration of symptoms at presentation was shorter in HIV infected patients (14 days versus 30 days, p<0.001). Headache was a more frequent symptom in HIV infected patients (97.0% versus 71.9%, p<0.001), and focal neurological symptoms were more common in HIV uninfected patients (40.35% versus 21.0%, p=0.028). There were no differences in the presence of the symptoms fever, neck stiffness, confusion, coma or fits between groups.

Table 3.3

Demographic and Historical Findings on Admission HIV Uninfected versus HIV Infected patients

Finding	HIV Uninfected		HIV Infected		Comparison
	N	Median (IQR) or N (%)	N	Median (IQR) or N (%)	P-value
Age (years)	57	37 (23, 51)	67	26 (23.5, 29.5)	0.002
Male Gender	57	31 (54.4%)	67	59 (88.1%)	< 0.001
Length of History (days)	57	30 (10, 38)	61	14 (7, 21)	< 0.001
Fever	57	44 (77.2%)	66	53 (80.3%)	0.83
Headache	57	41 (71.9%)	66	64 (97.0%)	< 0.001
Neck Stiffness	57	49 (86.0%)	60	45 (75.0%)	0.17
Confusion	57	20 (35.1%)	67	26 (38.8%)	0.71
Coma	57	14 (24.6%)	67	9 (13.4%)	0.16
Fits	57	7 (12.3%)	64	9 (14.1%)	0.80
Focal Neurological Symptoms	57	23 (40.4%)	62	13 (21.0%)	0.03

3.4.2.2 Examination findings

Examination findings are shown below in table 3.4. There were differences detected in distribution of both systolic and diastolic blood pressure between patient groups.

Table 3.4

Admission Findings on Examination HIV Uninfected versus HIV Infected patients.

Finding	HIV Uninfected		HIV Infected		Test
	N	Median (IQR) or N (%)	N	Median (IQR) or N (%)	P-Value
Weight (kilo)	26	45.5 (38.3, 52)	66	45.0 (40.3, 50)	0.96
Temperature (C)	57	37.5 (37, 38)	66	37.6 (37, 38.2)	0.62
Pulse (bpm)	57	90 (80, 100)	66	90 (80, 100)	0.56
Systolic Blood Pressure (mmHg)	57	120 (106, 130)	66	110 (100, 120)	0.005
Diastolic Blood Pressure (mmHg)	57	70 (60, 80)	66	70 (60, 80)	0.03
Glasgow Coma Score	57	15 (14,15)	66	15 (13, 15)	0.80
Cranial Nerve lesion	57	24 (42.11)	67	22 (32.84)	0.35
Papilloedema	57	22 (38.6)	59	12 (20.3)	0.04
Visual Impairment	53	23 (43.4)	55	19 (34.6)	0.43
Photophobia	-	-	58	6 (10.3)	-
Neck Stiffness	57	49 (86.0)	66	49 (74.2)	0.12
Hemiplegia	57	3 (5.3)	67	1 (1.5)	0.33
Paraplegia	57	2 (3.5)	67	0 (0)	0.21
Urinary Retention	-	-	66	3 (4.6)	-
Lymphadenopathy	57	0 (0)	67	6(9.0)	0.03
Respiratory symptoms	57	2 (3.5)	64	4 (6.3)	0.68
Splenomegaly	57	0	67	0	1.0
Hepatomegaly	57	0	67	5 (7.5)	0.06
Oral thrush	-	-	66	44 (66.7)	-
Any Focal Sign	57	27 (47.4)	67	22 (32.8)	0.10

The median systolic blood pressure was 120 mmHg in HIV uninfected patients versus 110 in HIV patients ($p = 0.005$). Papilloedema occurred in 38.6% of HIV uninfected patients versus 20.3% of HIV infected patients ($p = 0.04$). Visual impairment was determined using bedside tests – presence of symptoms, the ability to read news print (if literate), finger counting, movement detection, light perception, and visual fields tests by confrontation. The rate of visual impairment was not different between groups (43.4% versus 34.6%, $p=0.43$). Lymphadenopathy was uncommon and more prevalent in HIV infected patients (0 versus 9.0%, $p = 0.03$). There were no statistically significant differences seen in the presence of neck stiffness, any focal neurological signs, cranial nerve lesions, hemi or paraplegia, Glasgow coma score or respiratory symptoms.

3.4.2.3 Laboratory investigations

The results of haematological investigations are given in Table 3.5 below. Total white cell count, neutrophil count and lymphocyte count were all lower in HIV infected patients ($p < 0.001$). The median CD4 count in HIV infected patients was 14.5 cells/uL. Serum bilirubin was slightly higher in HIV uninfected patients ($p = 0.043$). CD4 count was measured in 20 of the HIV negative patients. The median CD4 count was 416.5/uL, and 7 patients had CD4 counts less than 200 /uL.

There were no differences in the rates of positive cryptococcal antigen tests in blood between patients (100% versus 97.44%, $p = 0.47$).

The results of cerebrospinal fluid examination are shown in Table 3.6 below. There were no differences in CSF opening pressure between patient groups. Cell counts in cerebrospinal fluid from HIV infected patients were significantly lower than in HIV uninfected patients.

Table 3.5

Haematological Findings on Admission HIV Uninfected versus HIV Infected patients

Investigation	HIV Uninfected		HIV Infected		Test
	N	Median (IQR) or N (%)	N	Median (IQR) or N (%)	P Value
Haemoglobin (g/dl)	57	12.4 (11.2,13.9)	60	12.1 (10.15,13.45)	0.06
White cell count ($X10^3$ cells/uL)	55	10.5 (8.15,12.8)	62	7.3 (4.67,8.44)	< 0.001
Neutrophil count ($X10^3$ cells/uL)	52	8.39 (6.4,10.6)	62	5.72 (3.5,6.9)	< 0.001
Lymphocyte count ($X10^3$ cells/uL)	51	1.21 (0.7,1.6)	62	0.6 (0.4,1.02)	< 0.001
Platelets ($X10^3$ cells/uL)	57	237 (140,335)	62	200 (149,267)	0.17
Sodium (mmol/L)	51	133 (128.5,137.5)	62	134.1 (129.62,137)	0.55
Potassium (mmol/L)	51	3.7 (3.2,4.08)	62	3.63 (3.2,4.2)	0.99
Urea (mmol/L)	51	4.32 (3.55,6.4)	61	4.5 (3.9,6)	0.80
Creatinine (mmol/L)	56	85.6 (68.1,87.8)	61	80 (64,95)	0.46
AST (U/L)	47	37 (23,49)	58	38 (28,54.25)	0.75
ALT (U/L)	47	40 (27,68)	58	51.5 (34.5,74.8)	0.15
Bilirubin (mmol/L)	47	12 (8,16.5)	52	10 (8,12)	0.04
Cryptococcal antigen (positive)	44	44 (100%)	39	38 (97.4%)	0.47
CD4 (cells/uL)	20	416.5(53 – 494)	48	14.5 (8, 28)	<0.001

Table 3.6

Cerebrospinal Fluid Findings HIV Uninfected versus HIV Infected patients

Investigation	HIV Uninfected		HIV Infected		Comparison
	N	Median (IQR) or N (%)	N	Median (IQR) or N (%)	P value
Opening Pressure (cm/CSF)	54	36.5 (18.2 - >40)	53	27 (17 - >40)	0.33
Opening Pressure >20cmCSF	54	36 (66.67)	53	36 (67.92)	1.0
White cells (cells/mL)	57	147 (66, 250)	63	26 (4.5, 67.5)	< 0.001
Neutrophils (cells/mL)	53	40 (13, 144)	48	9.5 (2, 38.75)	< 0.001
Lymphocytes (cells/mL)	53	83 (40, 130)	49	30 (7, 67)	< 0.001
Red cell count (cells/mL)	55	20 (0.5, 180)	61	1 (0, 48)	0.08
Protein (g/L)	57	1.2 (0.85, 1.55)	64	0.7 (0.5, 1.2)	< 0.001
Lactate (mmol/L)	55	4.6 (3.3, 6.05)	54	2.65 (1.83, 3.5)	< 0.001
Glucose (mmol/L)	57	1.4 (1, 2)	64	2.3 (1.58, 3)	< 0.001
Blood glucose (mmol/L)	57	5.8 (5.2, 7.1)	63	5.4 (4.7, 6.29)	0.03
Blood:CSF glucose ratio	57	4.35 (3.23, 7.14)	62	2.33 (1.86, 3.15)	< 0.001
Culture positive	57	56 (98.3%)	67	67 (100%)	0.46
Log2 *Cryptococcus* Antigen titre	52	8 (5, 10)	65	11 (9 – 13)	<0.001
Log 10 *Cryptococcus* colony forming units/ml	11	5.22 (4.37, 6.10)	48	6.02 (5.63, 6.61)	0.02

In HIV infected patients CSF protein and lactate were significantly lower (median 0.7 versus1.2 g/L, p<0.001 and 2.65 versus 4.6 mmol/L, p < 0.001), and the blood:CSF glucose ratio was smaller (p < 0.001). The fungal load was higher in HIV infected patients compared with HIV uninfected patients as measured by both the CSF cryptococcal antigen titre (1/2048 versus 1/256, p < 0.001) and the CSF viable cryptococcal count (1 050 000 CFU/ml versus 166 000 CFY/ml, p = 0.02). Log2 CSF cryptococcal antigen titre and quantitative fungal count were correlated for each group: Spearman's rank correlation was 0.49, p<0.001 for HIV infected patients and 0.61, p=0.04 for HIV uninfected patients.

3.4.2.4 Radiological Findings

21.4% of HIV uninfected patients had abnormalities on chest X-ray compared with 38.9% of HIV infected patients (p = 0.06). Infiltrative shadowing was the most frequently reported finding. One patient had bilateral pleural effusions; cavitation was not described. Overall only 39 patients had CT brain scans. 65.4% of HIV uninfected patients had abnormal scans, compared with 30.8% of HIV infected patients (p=0.09) (Table 3.7).

Table 3.7

Abnormal Radiological Findings HIV Uninfected versus HIV Infected

Characteristic	HIV Uninfected		HIV Infected		Comparison
	N	Summary Statistic	N	Summary statistic	P value
Chest X-ray	56	12 (21.43%)	54	21 (38.9%)	0.06
CT brain scan	26	17 (65.4%)	13	4 (30.8%)	0.09

3.4.2.5 Microbiology

Isolates were available from 60 of the HIV infected patients and 37 of the HIV uninfected patients. All isolates from HIV infected patients were *Cryptococcus neoformans* var *grubii* (*URA5* RFLP molecular group VN1). 11 (29.7%) isolates from HIV uninfected patients were *C. neoformans* var *gattii* (9 *URA5* RFLP group VG1 and 2 *URA5* RFLP group VG2); the rest (26, 70.3%) were *Cryptococcus neoformans* var *grubii,* all of molecular group VN1. This difference in species distribution was highly significant ($p<0.001$, Fishers' exact test). Of the HIV uninfected patients, 2 of 11 (18%) with *C. gattii* infection had underlying disease, versus 9 of 26 (34%) patients with var *grubii* infection ($p = 0.45$, Fisher's exact test). Representative RFLP profiles are shown in Figure 3.8.

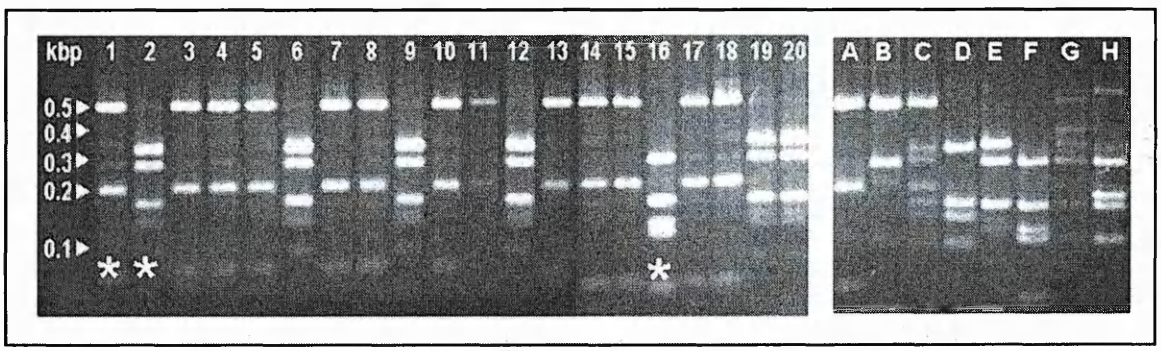


Figure 3.8.Representative PCR-RFLP profiles for the URA5 gene of 20 isolates and 8 controls. A = VN1, B = VN2, C = VN3, D = VN4, E = VG1, F = VG2, G = VG3, H = VG4. * identifies different molecular groups.

3.4.2.6 Mortality

The survival curves are shown in Figure 3.9.

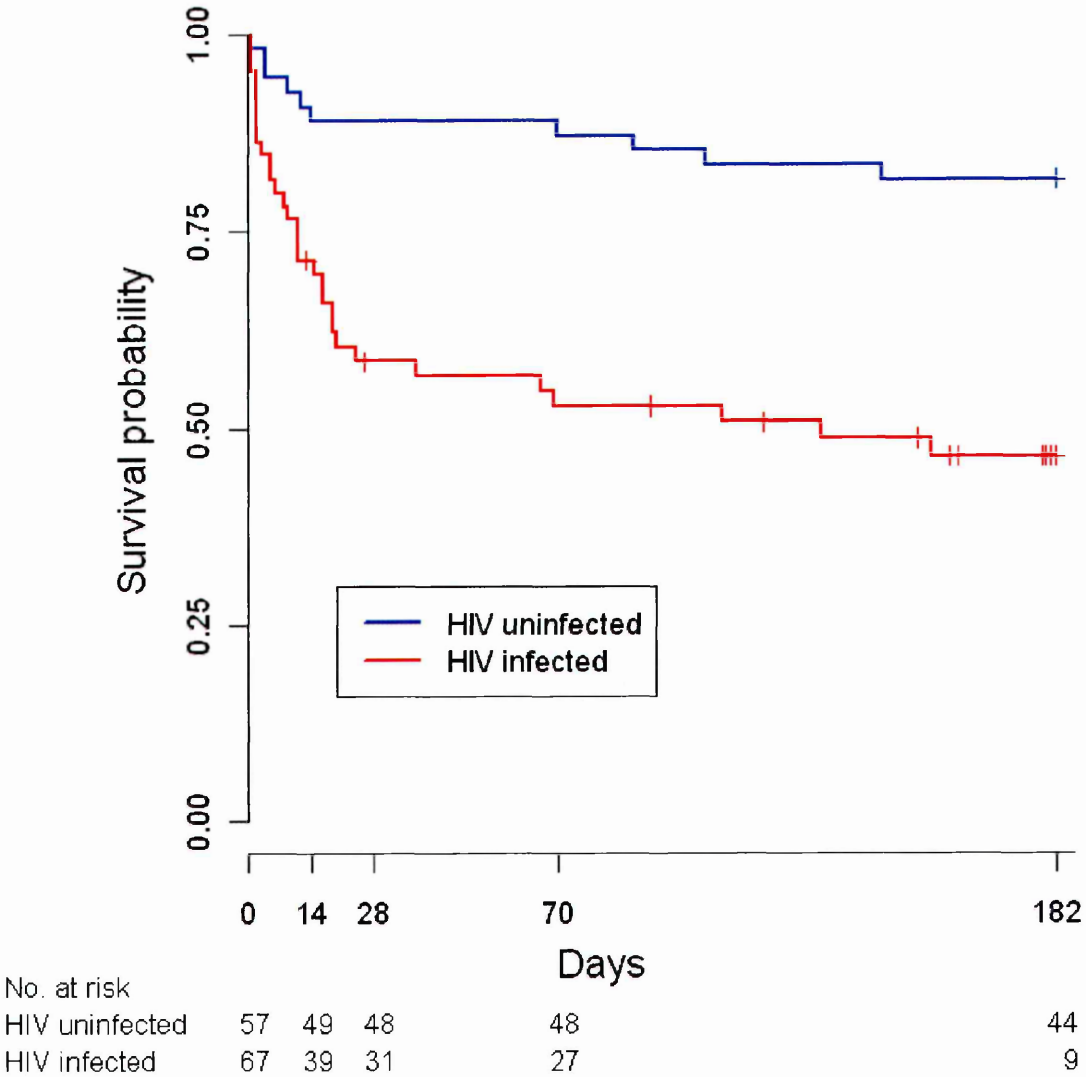


Figure 3.9.Six month Kaplan – Meier survival curve for HIV infected and uninfected patients with cryptococcal meningitis.

By 6 months 41 of 124 patients had died (33.1%). 2 HIV infected patients were lost to follow up, and 10 had withdrawn from the study. There was no loss to follow-up of HIV uninfected patients. The differential study withdrawal rate between the HIV serostatus

groups likely represents their difficult social circumstances (including high rates of drug abuse). 31 deaths occurred in HIV infected patients (46.3%) and 10 in HIV uninfected patients (17.5%). HIV infection increased the hazard for death within 6 months by a factor of 3.78 (95% confidence interval (CI) 1.841 - 7.734, $p<0.001$). By 10 weeks there were 35 deaths in total (28.2%), 7 occurring in the HIV uninfected group (death rate 12.3%) and 28 in the HIV infected group (death rate 41.8%). HIV infection increased the hazard for death by 10 weeks 4.5 fold (95% confidence interval 1.96 - 10.34, $p = 0.0004$). One of 11 HIV uninfected patients with var *gattii* infection died versus 6 of 26 with var *grubii* infection (Odds ratio 2.93, 95% CI 0.29 – 151.46, $p = 0.65$, Fisher's exact test).

3.4.3 Prognostic Factors

The literature search identified 25 papers which described prognostic factors in cryptococcal meningitis [53, 61, 62, 80, 99-104, 106, 108, 155-157, 204, 255-257, 259, 260, 262, 264, 265]. Over 40 prognostic factors associated with death were identified, listed in Tables 3.8 a and b.

Table 3.8

Table 3.8a. Previously reported prognostic factors in cryptococcal meningitis (history and examination findings)

	Prognostic Factor	Number of Studies Reporting Factor	Univariate Significance	Multivariate Significance
Clinical History and Examination	Mini Mental Test Examination score< 25	1	Y	Y
	Weight <55 kilo	1	N	Y
	Weight <40 kilo	1	Y	
	Male gender	2	Y	Y
	Age	2	Y	Y
	Age > 30yrs	1	Y	
	Underlying Disease	1	Y	
	Haematological Malignancy	3	Y	Y
	Disseminated Infection	3	Y	Y
	Malignancy	2	Y	Y
	Diabetes Mellitus	1	Y	
	Hepatitis B Infection	1	Y	
	AIDS	1		Y
	Renal Failure	1		Y
	Systolic Blood Pressure >150 mmHg	2	Y	
	Abnormal Mental Status	8	Y	Y
	'Cerebral Dysfunction' (GCS< 15 or Seizures)	1	Y	Y
	GCS < 13	1	Y	Y
	GCS <10	1	Y	
	Coma	1	Y	
	Abnormal Neurology*	3	Y	N
	Convulsion	3	Y	
	Blurred vision	1	Y	
	Absence of Headache	3	Y	Y
	Hydrocephalus	1	Y	
	Organ Failure	1	Y	Y
	Mechanical Ventilation	1	Y	
	Previous ITU admission	1	Y	

* Abnormal neurology = any reported neurological deficit (eg hemiplegia, cranial nerve lesion etc)

Table 3.8b. Previously reported prognostic factors in cryptococcal meningitis (investigations)

		Prognostic Factor	Number of Studies Reporting Factor	Univariate Significance	Multivariate Significance
Haematological	Findings	Fungaemia	3	Y	
		Cryptococcal Antigen Titre > 1/512	1	Y	Y
		Cryptococcal Antigen Titre	1	Y	
		Low haematocrit	1	Y	
		White Cell Count > 10 000/ml	1	Y	
		Thrombocytopenia	1	Y	
		Hyponatraemia	2	Y	Y
		High Blood Urea Nitrogen	1	Y	
		Low albumin	1	Y	Y
Cerebrospinal Fluid Examination		High CSF Pressure	2	Y	
		Opening pressure > 60 cm CSF	1	Y	
		Opening Pressure > 30 cm CSF	1	Y	Y
		Positive India Ink	2	Y	
		Cryptococcal colony Forming Units/ml CSF	2	Y	Y
		High CSF Cryptococcal antigen titre	1	Y	
		Cryptococcal Antigen titre > 1/512	1		Y
		Cryptococcal Antigen titre > 1/1024	2	Y	Y
		Low white cell count	1	Y	
		White Cell Count $<0.02X10^9/L$	3	Y	Y
		High CSF protein	1	Y	
		Protein<0.35g/L	1	Y	Y
		Low CSF Glucose	1	Y	
		CSF Glucose < 2mmol/L	1	Y	
Imaging		Abnormal Brain Imaging	3	Y	
		Abnormal Thoracic Imaging	1	Y	

3.4.3.1 Univariate Analysis

A Cox regression survival analysis was used to determine the validity of these factors in our dataset, both for all patients combined and separated by HIV serostatus, over both 70 days (the end of intensive treatment and the point at which patients are switched to long term suppressive therapy) and 6 months. The results of univariate analyses are summarised in Tables 3.9 – 3.13. The factors significantly associated with outcome are summarised in Table 3.14. For the combined group 6 factors (HIV infection, confusion, Glasgow Coma Score, cerebrospinal fluid cryptococcal antigen titre, cerebrospinal fluid colony forming unit quantitative count, and CSF white cell count) were associated with an increased hazard of death by both 70 days and 6 months. In addition, an abnormal chest X-ray was associated with death at 6 months. A low CSF glucose was associated with a reduced hazard of death, contrary to the published literature, perhaps indicating a robust immune response. For HIV patients, male sex was associated with a reduced hazard of death by both 70 days and 6 months, and presence of focal neurological symptoms with death by 6 months. For HIV uninfected patients, age, hemiplegia and blood urea were associated with death by both 70 days and 6 months.

Table 3.9 **Univariate Cox Regression of Demographic and Symptom Related Prognostic factors**

Variable (N: all, HIV+, HIV-)	**70 day outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value**			**6 month outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value**		
	All Patients	**HIV infected**	**HIV uninfected**	**All Patients**	**HIV infected**	**HIV uninfected**
Gender (male) (124, 67, 57)	0.947 [0.46 - 1.97] 0.88	**0.165 [0.07 -0. 41] <0.0001**	1.155 [0.26 - 5.16] 0.85	1.056 [0.53 - 2.11] 0.88	**0.165 [0.06 - 0.41] <0.0001**	1.31 [0.37 - 4.64] 0.68
Age + 10 years (124, 67, 57)	1.0 [0.78 - 1.28] 0.98	1.0 [0.59 – 1.69] 0.99	**1.66 [1.06 – 2.60] 0.03**	1.08 [0.86 - 1.34] 0.52	1.02 [0.63 - 1.68] 0.93	**1.77 [1.21 – 2.58] 0.003**
Length of History (+ 1 day) (118, 61, 57)	0.984 [0.96 - 1.00] 0.15	1.007 [0.98 - 1.04] 0.64	0.9823 [0.94 - 1.02 0.39	0.991 [0.98 - 1.01] 0.30	1.009 [0.98 - 1.04] 0.55	0.997 [0.98 - 1.01] 0.69
HIV status (positive) (124)	**4.50 [1.96 - 10.34] 0.0004**			**3.773 [1.84 - 7.73] 0.0003**		
Headache (124, 67, 57)	6.364 [0.87 - 46.55] 0.07	All have headache	2.165 [0.26 - 17.98] 0.47	2.487 [0.77 - 8.08] 0.129	All have headache	0.855 [0.221 - 3.31] 0.82
Confusion (124, 67, 57))	**1.962 [1.01 - 3.81] 0.05**	1.166 [0.55 - 2.47] 0.69	**12.72 [1.53 - 105.8] 0.02**	**1.859 [1.01 - 3.44] 0.05**	1.017 [0.49 - 1.89] 0.96	**9.312 [1.97 - 43.99] 0.005**
Convulsion (121, 64, 57)	1.555 [0.64 - 3.77] 0.33	1.051 [0.36 - 3.05] 0.93	3.357 [0.65 - 17.33] 0.15	1.53 [0.68 - 3.47] 0.31	0.894 [0.311 - 2.57] 0.84	3.748 [0.97 -14.52] 0.06
Focal Neurological Symptoms (119, 62, 57)	0.9041 [0.42 - 1.96] 0.80	2.067 [0.86 - 4.98] 0.11	0.556 [0.11 - 2.86] 0.47	1.252 [0.64 - 2.46] 0.52	**2.31 [1.01 - 5.31] 0.049**	1.399 [0.41 - 4.84] 0.60

Table 3.10 **Univariate Cox Regression of Physical Examination Predictors**

Variable (N: all, HIV +, HIV-)	**70 day outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value**			**6 month outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value**		
	All Patients	**HIV-Positive**	**HIV-negative**	**All Patients**	**HIV-Positive**	**HIV-negative**
Weight<40kg (92, 66, 26)	0.3432 [0.08 - 1.44] 0.14	0.374 [0.05 - 2.76] 0.33	0.793 [0.08 - 7.63] 0.84	0.0.29 [0.07 - 1.22] 0.09	0.334 [0.05 - 2.45] 0.28	0.582 [0.07 - 5.211] 0.63
Systolic BP>150mmHg (123, 66, 57)	1.332 [0.18 - 9.75] 0.78	1.428 [0.19 - 10.53] 0.73	8.059e-07 [0 - Inf] 1.0	1.192 [0.16 - 8.69] 0.86	1.428 [0.19 - 10.53] 0.73	8.05e-07 [0 - Inf] 1.0
GCS (+1) (123, 66, 57)	**0.860 [0.78 - 0.95] 0.002**	0.884 [0.77 - 1.01] 0.07	**0.767 [0.66 - 0.90] 0.001**	**0.876 [0.80 - 0.96] 0.004**	0.903 [0.79 - 1.03] 0.13	**0.801 [0.70 - 0.919] 0.002**
GCS<15 (123, 66, 57)	**2.237 [1.15 - 4.34] 0.02**	1.524 [0.72 - 3.22] 0.27	**12.72 1.53 - 105.8] 0.02**	**2.143 1.16 - 3.96] 0.02**	1.37 [0.66 - 2.83] 0.40	**9.312 [1.97 - 43.99] 0.005**
Cranial nerve lesion (124)	0.960 [0.48 - 1.91] 0.91	1.429 [0.67 - 3.05] 0.36	0.513 [0.10 - 2.65] 0.41	1.283 [0.69 - 2.38] 0.43	1.643 [0.80 - 3.36] 0.17	1.288 [0.37 - 4.45] 0.69
Blurred Vision (108, 55, 53)	0.659 [0.30 - 1.47] 0.31	0.822 [0.33 - 2.06] 0.68	0.472 [0.09 - 2.43 0.37	0.556 [0.27 - 1.17] 0.12	0.795 0.34 - 1.88] 0.60	0.289 [0.06 - 1.36] 0.12
Hemiplegia (124)	2.798 [0.86 - 9.14] 0.10	2.055 [0.28 - 15.25] 0.48	**8.42 [1.63 - 43.55] 0.01**	2.579 [0.79 - 8.38] 0.12	2.055 [0.28 - 15.25] 0.48	**6.371 [1.33 - 30.44] 0.02**
Any Focal Sign (124)	0.980 [0.50 - 1.93] 0.95	1.429 [0.67 - 3.05] 0.36	0.801 [0.18 - 3.58] 0.77	1.277 [0.69 - 2.36] 0.44	1.643 [0.80 - 3.36] 0.17	1.613 [0.46 - 5.72] 0.46

Table 3.11 **Univariate Cox Regression of Blood Investigation Related Predictors**

Variable (All, HIV+, HIV-)	70 Day Outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value			6 Month Outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value		
	All Patients	**HIV-Positive**	**HIV-negative**	**All Patients**	**HIV-Positive**	**HIV-negative**
WCC +log10 (10^3/uL) (117, 62, 55)	0.48 [0.14 - 1.62] 0.24	2.62 [0.34 - 20.1] 0.36	0.62 [0.05 - 8.41] 0.72	0.63 [0.20 - 1.05] 0.46	3.22 [0.46 - 22.7] 0.24	1.34 [0.08 - 22.3] 0.84
WCC >10 *10^3/uL (117, 62, 55)	0.43 [0.18 - 1.03] 0.06	1.17 [0.35 - 3.89] 0.80	0.61 [0.14 - 2.74] 0.52	0.56 [0.26 - 1.17] 0.12	1.48 [0.52 - 4.26] 0.47	0.81 [0.23 - 2.78] 0.73
Sodium (by+1mmol/L)	1.01 [0.96 – 1.07] 0.65	1.03 [0.96 – 1.10] 0.42	0.98 [0.88 – 1.09] 0.76	1.02 [0.97 – 1.07] 0.48	1.03 [0.97 – 1.10] 0.34	1.0 [0.91 – 1.09] 0.95
Sodium <130 mmol/L (113, 62, 51)	1.32 [0.64 - 2.71] 0.45	1.23 [0.54 - 2.81] 0.63	1.96 [0.44 - 8.76] 0.38	1.10 [0.55 - 2.20] 0.80	1.11 [0.49 - 2.49] 0.81	1.30 [0.33 - 5.19] 0.71
Urea (+log10 mmol/L) (117, 61, 56)	2.37 [0.34 - 16.68] 0.39	0.78 [0.08 - 7.27] 0.83	**90.65 [1.99 - 4136] 0.02**	2.09 [0.34 - 12.92] 0.43	0.74 [0.09 - 6.20] 0.78	**41.7 [1.32 - 1319] 0.034**
Urea >6.7 mmol/L	1.11 [0.46 - 2.69] 0.82	1.03 [0.35 - 2.99] 0.96	1.78 [0.35 - 9.18] 0.49	1.13 [0.50 - 2.56] 0.78	0.92 [0.32 - 2.64] 0.88	2.20 [0.55 - 8.78] 0.27
Cr (by+log10 mmol/L) (117, 61, 56)	3.04 [0.15 - 62.6] 0.47	1.20 [0.05 - 30.93] 0.91	246.8 [0.26–2*10^5] 0.12	1.72 [0.10 - 28.43] 0.71	0.73 [0.03 - 16.36] 0.84	39.96 [0.12 - 12944] 0.21
Positive Blood culture (28, 28, 0)		1.84 [0.23 - 14.53] 0.56			2.154 [0.28 - 16.72] 0.46	
HBsAg positive (40, 40, 0)		1.56 [0.44 - 5.47] 0.49			1.28 [0.37 - 4.40] 0.70	
CD4 count (by+ log10 cells/uL) (48)		0.57 [0.17 - 1.85] 0.34			0.57 [0.30 - 1.07] 0.08	

Table 3.12 Univariate Cox Regression of Cerebrospinal Fluid Associated Predictors

Variable (N: all, HIV+ , HIV-)	70 day outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value			6 month outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value		
	All Patients	**HIV-Positive**	**HIV-negative**	**All Patients**	**HIV-Positive**	**HIV-negative**
CSF Opening Pressure >40 cm CSF (107, 53, 54)	0.79 [0.38 - 1.64] 0.52	1.04 [0.44 - 2.43] 0.94	0.81 [0.18 - 3.63] P0.79	0.77 [0.38 - 1.56] 0.47	0.97 [0.42 - 2.25] 0.94	0.87 [0.23 - 3.25] 0.84
CSF OP>30 cm CSF (107, 53, 54)	0.83 [0.41 - 1.67] 0.59	1.14 [0.51 - 2.56] 0.74	0.82 [0.18 - 3.68] 0.80	0.87 [0.45 - 1.71] 0.69	1.06 [0.48 - 2.33] 0.89	1.25 [0.31 - 4.98] 0.76
CSF OP>20 cmCSF (107, 53, 54)	0.97 [0.46 - 2.06] 0.93	0.93 [0.40 - 2.17] 0.87	1.14 [0.22 - 5.86] 0.88	1.11 [0.53 - 2.32] 0.79	0.98 [0.42 - 2.28] 0.97	1.61 [0.33 - 7.73 0.56
Log2CSF CrAg (117,65,52)	**1.20 [1.08 - 1.34] 0.001**	1.07 [0.94 - 1.23] 0.30	**1.29 [1.03 - 1.63] 0.03**	**1.22 [1.10 - 1.35] <0.001**	1.08 [0.95 - 1.24] 0.24	**1.31 [1.08 - 1.59] 0.007**
Log10 QC CSF (CFU/ml) (58, 47,11)	**2.31 [1.24 - 4.30] 0.008**	1.97 [0.96 - 4.03] 0.07	4.16 [0.76 - 22.7] 0.10	**2.04 [1.16 - 3.57] 0.013**	1.64 [0.85 - 3.14] 0.14	4.16 [0.76 - 22.65] 0.10
Log10 QC>4.7 (58, 47, 11)	3.70 [0.50 - 27.58] 0.20	0.91 [0.12 - 6.89] 0.93	1X10^8 [0 - Inf] 0.99	4.49 [0.61 - 33.32] 0.14	1.07 [0.14 - 7.80] 0.95	11236498 [0 - Inf] 0.99
Log10 CSF WCC/ml (120, 63, 57)	**0.54 [0.36 - 0.82] 0.004**	0.72 [0.43 - 1.20] 0.21	1.36 [0.29 - 6.48] 0.70	**0.55 [0.37 - 0.81] 0.003**	0.70 [0.43 - 1.14] 0.15	1.31 [0.35 - 4.86] 0.69
Protein CSF/0.1 g/L (121, 64, 57)	0.98 [0.92 - 1.04] 0.41	1.00 [0.93 - 1.06] 0.91	1.03 [0.92 - 1.16] 0.59	0.97 [0.92 - 1.03] 0.34	0.99 [0.93 - 1.05] 0.70	1.04 [0.94 - 1.14] 0.47
Protein <0.35g/L (121, 64, 57)	1.27 [0.31 - 5.31] 0.74	0.71 [0.167 - 2.99] 0.64	All values greater than 0.35g/L	1.8 [0.26 - 4.47] 0.92	0.61 [0.15 - 2.58] 0.51	All values greater than 0.35g/L
Log10 Glucose CSF mmol/L (121, 64, 57)	**7.06 [1.75 - 28.44] 0.006**	1.96 [0.33 – 11.8] 0.46	**134.2 [1.11 – 16211] 0.05**	**6.51 [1.70 - 25.0] 0.006**	2.14 [0.39 – 11.7] 0.38	21.0 [0.37 – 1285] 0.14
Glucose<2.0 mmol/L (121, 64, 57)	**0.50 [0.25 - 0.99] 0.05**	0.95 [0.44 - 2.08] 0.90	0.32 [0.07 - 1.41] 0.13	0.59 [0.31 - 1.10] 0.10	0.96 [0.45 - 2.01] 0.91	0.62 [0.17 - 2.19] 0.46

Table 3.13 **Univariate Cox Regression of Imaging Findings and Outcome**

Variable (N all, N HIV+, N HIV-)	70 day outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value			6 month outcome Hazard Ratio [Lower 95% CI - Upper 95% CI] P value		
	All Patients	HIV-Positive	HIV-negative	All Patients	HIV-Positive	HIV-negative
Normal Chest X-ray (110, 54, 56)	0.59 [0.28 - 1.23] 0.16	0.76 [0.33 - 1.74] 0.52	0.67 [0.13 - 3.47] 0.64	**0.47** **[0.25 - 0.92]** **0.03**	0.67 [0.31 - 1.45] 0.31	0.40 [0.11 - 1.40] 0.15
Normal CT Brain Scan (39, 13, 26)	1.43 [0.32 - 3.38] 0.64	0.75 [0.14 - 4.11] 0.74	1.399e-07 [0 - Inf] 0.99	1.38 [0.37 - 5.15] 0.63	0.75 [0.14 - 4.11] 0.74	0.78 [0.07 - 8.57] 0.84

Table 3.14 **Summary of variables found to be associated with outcome by univariate analysis.**

Variable	N	70 Day Survival Hazard Ratio	95% Confidence Interval	P value	6 Month Survival Hazard Ratio	95% Confidence Interval	P value
Confusion	124	1.96	1.01 - 3.81	0.046	1.86	1.01 - 3.44	0.048
Glasgow Coma Score (+1)	123	0.86	0.78 - 0.95	0.0019	0.88	0.80 - 0.96	0.004
Log_{10} CSF WCC/ml	120	0.54	0.35 – 0.82	0.004	0.55	0.37 – 0.81	0.003
HIV Infected	124	4.50	1.96 – 10.34	0.0004	3.77	1.84 – 7.73	0.0003
Log_2 CSF CrAg titre	117	1.20	1.0 - 1.34	0.001	1.22	1.10 – 1.35	0.0002
Log_{10} CSF Yeast Count	58	2.31	1.24 - 4.295	0.008	2.04	1.16 – 3.57	0.013
Log_{10} CSF Glucose mmol/L	121	7.06	1.75 - 28.44	0.006	6.51	1.70 - 24.98	0.006
Normal Chest X-ray	110	NA			0.47	0.25 0.92	0.026

3.4.3.1 Multivariate analysis

To be included in the multivariate analysis variables, the p-value of the univariate analysis had to be ≤0.2. In addition, in order not to decrease sample size too dramatically, data on the variable had to exist for at least 80% of the subjects. Because of limitations imposed by the sample size and the number of events, the multivariate model was only fitted to the 6 month time point for the whole group of patients. Table 3.15 summarises the variables where $0.05<P\leq0.2$ over 6 months for the whole patient group.

Table 3.15

Summary of variables associated with death with P values $0.05 < P \leq 0.2$ (all patients).

Variable	6 Month Survival			
	N	Hazard Ratio	95% CI	P value (Wald)
Weight <40 kilo	92	0.29	0.07 – 1.217	0.091
Hemiplegia	124	2.579	0.794 - 8.375	0.115
Blurred Vision	108	0.556	0.265 - 1.17	0.122
Headache	124	2.487	0.766 - 8.076	0.129
Blood WCC>10 *10^3 cells/uL	117	0.555	0.263 - 1.169	0.121

WCC = white cell count

CSF yeast quantitative counts and weight data were only available for 58 (47%) and 92 (74%) patients. Thus eleven variables met the inclusion criteria for the final model – HIV serostatus, headache, confusion, blurred vision, hemiplegia, Glasgow coma score, CSF glucose, CSF cryptococcal antigen titre, CSF white cell count, blood white cell count and abnormalities on chest X-ray and the dataset with complete data on all these variables included 99 patients. After backward selection, 5 covariates remained in the model for prediction of death at 6 months (Table 3.16).

Table 3.16

Multivariate Analysis - Final Model (99 patients)

Variable	Hazard Ratio	Lower 95 CI	Upper 95 CI	P value
Log_2 CrAg CSF (+1 log_2)	1.249	1.10	1.42	<0.001
GCS (+1)	0.839	0.74	0.96	0.007
Hemiplegia	3.76	0.80	17.61	0.093
Abnormal Chest X-ray	1.83	0.89	3.74	0.098
CSF WCC (+ 1Log_{10}cells/ml)	0.53	0.33	0.85	0.008

CrAg = cryptococcal antigen titre, GCS = Glasgow Coma Score, WCC = white cell count

Glasgow coma score, cryptococcal antigen titre in CSF and CSF white cell count were highly significantly associated with an increased hazard of death. Refitting the variables identified by the model on all subjects with complete data increased the model size to

104 patients. The same variables remained significantly associated with death (Table 3.17).

Table 3.17

Effect of refitting the multivariate model on all patients with observations on the selected covariates (104 patients)

Variable	Hazard Ratio	Lower 95 CI	Upper 95 CI	P value
Log_2 CrAg CSF (+1 log_2)	1.23	1.09	1.38	<0.001
GCS (+1)	0.81	0.73	0.92	<0.001
Hemiplegia	3.27	0.72	14.94	0.126
Abnormal Chest X-ray	1.82	0.92	3.61	0.087
CSF WCC (+ 1Log_{10}cells/ml)	0.47	0.31	0.73	<0.001

CrAg = cryptococcal antigen titre, GCS = Glasgow Coma Score, WCC = white cell count

Because of the differences in mortality rate between HIV infected and uninfected patients, the backward selection was repeated but forcing HIV status into the model. Glasgow coma score and CSF cryptococcal antigen titre remained significantly associated with death (Tables 3.18 and 3.19).

Table 3.18

Multivariate analysis - final model, HIV status forced into model (99 patients)

Variable	Hazard Ratio	Lower 95 CI	Upper 95 CI	P value
Log2CrAg CSF	1.226	1.07	1.40	0.003
Glasgow Coma Score	0.842	0.74	0.96	0.009
Hemiplegia	3.99	0.85	18.83	0.080
Abnormal Chest X-ray	1.89	0.92	3.90	0.083
Log10 CSF WCC	0.60	0.34	1.04	0.068
HIV infected	1.46	0.54	3.91	0.45

WCC = white cell count

Table 3.19

Refitting the model on all patients with observations, + HIV status (104 patients)

Variable	Hazard Ratio	Lower 95 CI	Upper 95 CI	P value
Log2CrAg CSF	1.20	1.06	1.37	0.004
Glasgow Coma Score	0.82	0.73	0.93	0.001
Hemiplegia	3.52	0.77	16.15	0.106
Abnormal Chest X-ray	1.88	0.0.95	3.76	0.072
Log10 CSF WCC	0.54	0.32	0.90	0.019
HIV infected	1.54	0.58	4.04	0.385

In a 100 iteration boot strap analysis all 5 variables initially chosen by the model were reselected at least 50% of the time (Table 3.20).

Table 3.20

Bootstrap Analysis of Final Model

Variable	Probability of the selected variable being included in the final model
Log2 Cryptococcal Antigen in CSF	0.92
Glasgow Coma Score	0.77
Log10 white cell count CSF	0.76
Hemiplegia	0.66
Abnormal Chest X-ray	0.54
Visual Impairment	0.49
Log10 blood white cell count	0.41
HIV status	0.41
CSF Glucose< 2 mmol/L	0.40
Headache	0.31
Confusion	0.27

3.5 Discussion

3.5.1 Published Clinical Phenotypes

There was heterogeneity in the groups of patients from whom descriptions of the clinical phenotype of cryptococcal meningitis have been published. This might have been expected for HIV uninfected patients, since their medical backgrounds are diverse, ranging from the solid organ recipient to the immunocompetent. More surprisingly the heterogeneity was at least as marked within the HIV infected population. This may have implications for the extrapolation of study findings from one patient group to another. Of note, van der Horst's study, which has been the key study underlying the development of treatment guidelines for cryptococcal meningitis, was remarkable for the relatively low proportion of patients suffering headache in contrast to other series[107, 110]. Since headache can be a symptom of raised intra-cranial pressure, this may signify that patients in the study were less severely ill than the general population of patients with cryptococcal meningitis – ie they were not a representative sample. There were marked differences in the prevalence of neck stiffness and meningeal signs between studies. The absence of meningeal signs could conceivably be a marker of both mild and severe disease – absent in severe cases because of the inability of the patient to report it, or because of profoundly suppressed immunity and subsequent minimal inflammatory response. The differences in presence of symptoms at baseline may also represent health seeking behaviour that occurs at different disease stages because of differences in wealth or access – French describes patients from Uganda, whereas van der Horst reports patients from the USA. Of course, intervention studies are biased in their presentation of clinical symptoms and signs by their inclusion criteria, and purely descriptive studies,

with less stringent entry criteria, are more likely to give an accurate clinical phenotype. However, a clinical trial where the study population does not accurately reflect the general population is of limited value.

The intra-group (HIV infected or not) heterogeneity that I have demonstrated suggests that my data concatenation must be interpreted with a deal of caution. My concatenation suggests that there are significant differences in clinical phenotype between HIV infected and uninfected patients, regarding the presence of confusion/impaired conscious level, focal neurological signs, fits, visual impairment and nausea and vomiting. How this compares with the experience in Viet Nam is discussed below.

3.5.2 Clinical Phenotype in Viet Nam

3.5.2.1 History and Examination

I found statistically significant differences in historical findings but few differences in examination findings in patients with cryptococcal meningitis according to HIV serostatus. HIV infected patients were more likely to be male (88.1% versus 54.4%) and the median age at presentation was lower (26 years versus 37 years). The age and gender distribution in HIV infected patients mirrors the demographics of the HIV epidemic in Viet Nam. There was no gender preponderance in HIV uninfected patients (31 of 57 patients were male, 54.4%). The duration of illness prior to presentation in HIV infected patients was shorter than in HIV uninfected patients (14 versus 30 days). It might have been expected to be longer given that a high proportion of the HIV infected patients are intravenous drug users (67.75%) – a group that is generally considered to be in a socially disadvantaged position with impaired contact with health services. The shorter illness

duration presumably represents more rapid disease progression because of immunosuppression. Consistent with this, the markers of fungal burden (CSF cryptococcal antigen titres and yeast colony forming units) were significantly higher in HIV infected patients at presentation. However, despite the shorter disease duration and higher fungal burdens, physical examination findings, including Glasgow Coma Score, were similar in each group.

Headache, a symptom associated with raised intra-cranial pressure, was frequent in both groups but more commonly reported in HIV infected patients (97% versus 72%, $p<0.001$). However, papilloedema was marginally more common in HIV uninfected patients (38.6% versus 20.34%, $p = 0.04$). The different rates of papilloedema, which takes time to develop, perhaps represent the differences in symptom duration in each group – longer in the HIV uninfected. The median systolic and diastolic blood pressures were the same between groups, but the spread was different, with more HIV uninfected patients having higher values ($p = 0.005$). Other indicators of raised intracranial pressure, such as the presence of coma, pulse rate and the opening CSF pressure were no different between groups.

Lymphadenopathy was not reported in the HIV uninfected patients but was present in 9% of HIV infected patients, and is likely to be a manifestation of HIV rather than cryptococcosis per se.

While in these patients the prevalence of particular symptom/signs by HIV status were broadly similar to that shown in the data concatenation, I did not find the same statistically significant differences. This may be a function of study size. Of note, visual impairment was much higher in our HIV infected patients compared with the published

literature. Perhaps other factors were important, such as malnutrition or toxins related to intravenous drug use, which is prevalent in these patients. Unlike the concatenation, I found headache to be more common in HIV infected patients (97% vs. 72%, $p<0.001$).

3.5.2.2 Haematological Findings

As might be expected, HIV infected patients had markedly lower blood lymphocyte counts than HIV uninfected patients ($p<0.001$). The median neutrophil count was also significantly lower (5.72×10^3/uL, $p<0.001$) although still within the normal range. The median CD4 count was 14 cells/uL in HIV patients. Only 20 HIV uninfected patients had CD4 counts measured on admission and the median count was 416 cells/uL. 9 patients had CD4 counts less than 400 cells/uL and 7 patients had CD4 counts less than 200 cells/uL. While CD4 lymphopenia is a recognised risk factor for cryptococcosis, transient CD4 lymphopenia is a well recognised feature of some infections including cryptococcosis and tuberculosis. Which of these possibilities is the case in our patients is not clear, since the CD4 count was not measured serially, although one can be confident that it is not due to HIV infection. I could not demonstrate any increased hazard for death at 70 days or 6 months of a low CD4 count in HIV uninfected patients. Differences in total bilirubin (median 10 mmol/L versus 12 mmol/L, $p=0.043$) were clinically unimportant, and due to the number of comparisons (discussed in more detail below) also statistically insignificant.

3.5.2.3 CSF Examination Findings

There were significant differences in CSF examination findings consistent with an attenuated immune response in the HIV infected patients. Total white cell count, neutrophil count and lymphocyte count were all lower in HIV infected patients ($p<0.001$ for all variables). Other CSF markers of inflammation including protein and lactate were significantly higher in HIV uninfected patients, and CSF glucose was lower in HIV uninfected patients, consistent with other reports from the literature [104, 156, 266]. The median CSF:blood glucose ratio was 0.23 in HIV uninfected patients versus 0.43 in HIV patients ($p = 0.001$). A similar impact of HIV on CSF parameters has been described in tuberculous meningitis [267]. Of note, in HIV negative patients there was no correlation between blood CD4 count and CSF lymphocyte count (Spearman's coefficient -0.01, $p = 0.97$).

There have been few studies of the effect of HIV on the presentation of cryptococcal meningitis. Rozenbaum and Goncalves retrospectively compared 130 patients with cryptococcal meningitis from Brazil according to underlying immune status (HIV infected, underlying potentially immunosuppressive disease excluding HIV, and not immunosuppressed)[259]. In keeping with my findings, they found that papilloedema was significantly more common in HIV uninfected patients. They also found impaired consciousness, neck stiffness, and cranial nerve lesions to be statistically significantly more common in HIV uninfected patients compared with HIV infected patients (Table 3.17). Similarly, I found increased rates of neck stiffness and cranial nerve lesions in these HIV uninfected patients, although the differences did not reach statistical significance.

Lui et al reported 31 patients from Taiwan with cryptococcal meningitis of whom 7 had HIV and 8 had other potentially immunosuppressive co-morbidities[104]. Consistent with this experience they found that immunocompetent patients had more indolent disease (p=0.02), higher CSF white cell counts (p=0.03) and better clinical outcomes (p=0.04). Moosa and Coovadia reported 44 HIV infected patients and 21 HIV uninfected patients with cryptococcal meningitis from Durban, South Africa[257]. They also found HIV infected patients were more likely to have headache (p = 0.04), were younger (p=0.02), had higher CSF cryptococcal antigen titres and lower CSF white cell counts than HIV uninfected patients. The in-hospital mortality rate was 7 fold higher for HIV infected patients (64% versus 9%, p=0.0007). Moosa et al reported that HIV infected patients were more likely to be female (p=0.01), (consistent with the demographic differences in the HIV epidemic in South Africa compared with Viet Nam), and that HIV infected patients were more likely to have neck stiffness (p = 0.02). Focal neurological signs were present in 50% of their HIV infected patients and 29% of their HIV uninfected patients (p = 0.1), compared with 32.8% and 47.4% respectively in our patients (p = 0.1). 7% of their HIV infected patients had normal CSF examination compared with none of the HIV uninfected patients (p = 0.05).

Dromer et al reported 53 HIV uninfected patients and 177 HIV infected patients from France[156]. 187 patients had cryptococcal meningitis, and no difference was found in clinical presentation by HIV status. Of 53 HIV uninfected patients, co-existent morbidity was common – solid organ transplantation in 11, malignancies in 21, other potentially immunosuppressive conditions (diabetes, cirrhosis, sarcoidosis, corticosteroid use, idiopathic CD4 lymphopenia and hypogammaglobulinaemia) in 12 and no identified risk

factor in 9 (of whom 5 had disease limited to the skin). This contrasts with our HIV uninfected patients (all with meningitis), the majority of whom have no identified potentially immunosuppressive co-morbidities. Speed and Dunt reported 133 patients from Victoria, Australia, of which 96 had meningitis and 25 had HIV infection, but did not compare clinical phenotype by HIV infection status[76].

Conclusions drawn from studies directly comparing cryptococcal meningitis clinical phenotype by HIV serostatus are limited by the small sizes of the studies. Further data regarding clinical phenotype can be derived from other study types, focusing on either patient group, such as randomised controlled trials. I identified baseline clinical data published on 2204 patients from the medical literature [57, 61, 62, 99-107, 144, 154, 157, 254-261]. However, these data must be interpreted with caution. For example, the largest RCT on cryptococcal meningitis (381 patients) was the Mycoses Study Group and ACTG trial (Van der Horst et al) published in 1997[107]. This study on HIV infected patients had stringent exclusion criteria which served to exclude patients considered to be more severely ill. Patients who were comatose, unable to take oral medication, unable to provide consent or who had moderate or severe haematological, renal or hepatic dysfunction were excluded from the study. Thus any conclusions about clinical phenotype must be considered within this context of presumably milder disease. Of course, all patient sub-populations are potentially biased, not least those seen in our hospital, which serves a large geographical area. The distances some patients need to travel could act as a barrier preventing the most unwell patients from reaching our hospital, although the death rates in our patients are similar to those reported from elsewhere in Asia. While it is tempting to collate the data from the studies noted above,

and it is possible to describe differences by HIV serostatus (Table 3.18), tests for heterogeneity reveal that the patient populations from which the data are drawn (even amongst HIV patients) are indeed highly heterogeneous and that this data collation is therefore likely to be misleading. This is illustrated in Table 3.19 and the Forest plots in Figures 3.3 – 3.10.

The other confounder that should be considered is that of multiple testing. I compared 55 baseline variables – probably not dissimilar to most descriptive studies, and thus might have expected to find at least 3 variables different between groups at a statistical significance level of $p = 0.05$. A Bonferroni correction (which is conservative) would suggest that the null hypothesis should be rejected for a comparison only where the P values is (approximately) 0.001[268]. We found 14 variables with a P value <0.001 (which should thus be considered significant at the P=0.05 level). 13 were laboratory measurements where the differences are plausible given that they might be expected to be lower in immunosuppressed patients, and the other was the rate of presence of headache, which was more frequent in HIV infected patients. Possible explanations for the differential presence of headache are more rapid disease progression in HIV infected patients, with subsequently more rapid changes in CSF pressure (CSF pressure measurement was essentially 'clipped' at 40cm CSF because of the measuring limit of the manometers used, so we cannot be certain that there were no differences by measurement), a higher fungal burden resulting in higher CSF pressures, again undetected for the reasons just described, or reduced pain tolerance in HIV patients because of opiate withdrawal due to disability amongst IV drug users.

3.5.2.4 Microbiology

All HIV infected patients were infected with *Cryptococcus neoformans* var *grubii* and this is in line with expectations for HIV patients outside of Europe. Of the 37 viable strains from HIV uninfected patients, 11 were *Cryptococcus neoformans* var *gattii*, 9 belonging to *URA5* RFLP molecular group VG1 and 2 belonging to molecular group VG2. In this tropical location, it might have been expected that more cases in HIV uninfected patients would have been due to infection with *Cryptococcus gattii* [64, 76, 269]. The ratio of var *grubii* to var *gattii* isolates was 3:1. Var *grubii* infection, the commonest cause of cryptococcal meningitis worldwide since the HIV epidemic, most frequently occurs in patients with an underlying immunosuppressive condition[61, 62, 270, 271]. Most of the HIV uninfected patients (81%) had no concomitant disease associated with immunosuppression, although I did detect CD4 lymphopenia at presentation in 9 patients, 3 of whom did not have other underlying conditions. I found no difference in the rate of underlying disease according to infecting isolate, although this could be explained by the relatively small numbers in this study.

Historically, the disease phenotypes due to infection with var *grubii* and *C. gattii* are considered to be different. Henderson first proposed the hypothesis that inherent differences in pathobiology according to variety could result in different clinical phenotypes [272]. Subsequently both Speed and Mitchell found that *C. gattii* tends to cause disease in the immunocompetent and that infections are frequently complicated by focal parenchymal brain lesions which are associated with hydrocephalus, cranial nerve palsies and seizures [61, 76]. In addition, the history is prolonged in *C. gattii* infection, frank pulmonary involvement is more common, and the outcome is better. However,

these differences in phenotype might be explained by the host immune response rather than the pathogen.

The small number of *C. gattii* infections I identified means my study lacks power to detect differences by infecting species, and, as might be expected, I found no differences in clinical phenotype between the 2 infections. However, the number of *C. gattii* infections was small, yet the pattern of disease seen in the HIV uninfected cases mimicked that seen in case series of *C. gattii* infection published from Papua New Guinea, suggesting that host immune status is indeed important in determining clinical phenotype [62, 80]. Male gender has been identified as a risk factor for *Cryptococcus gattii* infection in Australian patients (most of whom are HIV uninfected). Of the 11 *C. gattii* infections in our HIV uninfected patients, 4 were in women.

The remaining 26 isolates from HIV uninfected patients, like all the isolates from the HIV infected patients, were all *C. grubii* of *URA5* PCR-RFLP molecular group VN1. An alternative explanation to the host immune status as the prime determinant of clinical phenotype hypothesis, and that would explain why our *C. grubii* predominantly infected HIV negative patients have clinical phenotypes similar to that in patients with *C. gattii* infection, is that disease in these patients is due to a distinct sub-group of *C. grubii* that shares pathogenic features more usually associated with *C. gattii*. A study from China has recently reported that *Cryptococcus* strains causing cryptococcal meningitis in HIV uninfected patients there are most frequently *C. grubii* VN1, and that there is remarkable genetic homogeneity between the isolates, perhaps suggesting the existence of a strain in Asia with particular propensity for infecting the immunocompetent[273]. In fact, the authors of the study suggest that in China most cases of cryptococcal meningitis seem to

be occurring in the immunocompetent. This is not so in Viet Nam, where the major burden of disease is in HIV positive patients, with more than 200 cases diagnosed per year in the Hospital for Tropical Diseases (unpublished data).The molecular epidemiology of the infecting strains in Vietnamese isolates will be discussed in more detail in Chapter 4. The results of antifungal sensitivity testing of the strains will be discussed in more detail in Chapter 5. Ultimately, it seems most plausible that disease phenotype is determined by both the specific pathogen's properties and the host's immune status.

3.5.2.5 Mortality

The 6 month death rate in these HIV infected patients was 46.3% (95% CI 34.9 – 58.1%), which is in line with death rates reported from other low income countries. In Africa mortality rates for patients on fluconazole treatment are as high as 100% at 6 months, with a median time to death of 19 days [258]. Mayanja-Kizzi reported 6 month death rates of 78% (95%CI 64.6% - 87.4%) in patients randomised to receive either fluconazole or fluconazole combined with flucytosine [144]. Longley reported a 10 week death rate of 54% (95%CI 41.6 – 66.6%) in patients receiving high dose fluconazole in south-western Uganda [274]. In Thailand, the in-hospital mortality rate in patients receiving 'high dose' amphotericin is 28% (median hospital stay 28 days, range 1 – 91); the death rate at 6 months is not clear, but the cumulative mortality rate at 12 months in this study was 76% [106]. Rozenbaum's study from Brazil found the mortality rate to be 65.7% (95%CI 53.7 – 75.9%) in patients with cryptococcal meningoencephalitis [259]. In Durban, the in hospital death rate for HIV infected patients was 64% (95%CI 48.8 – 76.3%) [257].

In high income countries, the death rate is probably lower. The largest clinical trial is the ACTG randomised controlled trial of amphotericin B in combination with flucytosine in cryptococcal meningitis. In this multicentre USA-based study, which excluded severely ill patients, patients were initially randomised to amphotericin B with or without additional flucytosine. If the patients improved on this regime, they were then re-randomised into the second part of the trial, either to treatment with itraconazole or fluconazole. It is difficult to estimate an overall 10 week death rate in this study because there were patients who were initially randomised but subsequently left the study because they did not meet the criteria for the second randomisation step. Of the 381 patients enrolled, 21 died within the first 2 weeks. However, only 306 were considered eligible to be randomised to the second stage of the study. Of the 54 patients who did not enter the second stage at least 27 had deteriorated or had drug toxicities and 21 withdrew or were lost to follow up. Counting just the deaths in patients who remained in the trial and the deaths in the first 2 weeks, the 10 week death rate was 10.1% (33/327), 95% CI 7.2 – 13.9%. There was no follow-up after 10 weeks. This low death rate contrasts with Larsen et al, a small study of 20 patients from the USA, where the death rate was 40% at 10 weeks (95% CI 21.8 – 61.4%) [142].

The 6 month death rate in the HIV uninfected patients in our study was 17.5% (95%CI 9.6 – 29.6%). Comparing death rates between studies in HIV uninfected patients with cryptococcal meningitis is complicated by the heterogeneity of the underlying patient groups, in particular the presence of underlying disease that itself has significant mortality. Most series include patients receiving non-steroid immunosuppressive drugs, and malignancies, most usually haematological [53, 64, 103, 104, 156, 259, 262, 264,

275]. However, since most studies are small, the 95% confidence intervals are wide, and overlap between series. In immunocompetent patients with *C. gattii* infection, Seaton reports a death rate of 34.1% (95%CI 25 – 44.5%) in Papua New Guinea [62]. *C. gattii* infection in immunocompetent patients in Australia is associated with a mortality rate of 15% (95%CI 5.5 – 34.2%)[61]. Rozenbaum found the death rate in Brazil to be 38.6% (95%CI 25.7 – 53.4%) in patients with no apparent underlying disease and 36.8% (95%CI 19.1 – 59.1%) in patients with underlying disease excluding HIV. The proportions of disease due to *C. gattii* or *C. grubii* were not described in this paper[259]. Lui reports death rates of 18.8% (95%CI 5.8 – 43.8%), 62.5% (95%CI 30.4 – 86.5%)and 28.6% (95%CI 7.6 – 64.8%) for immunocompetent patients, patients with underlying disease excluding HIV, and HIV infected patients respectively, although the numbers in each group were small [104]. Shih found an overall death rate of 19.2% (95% CI 12.4 to 28.3 %) in 94 HIV uninfected patients with meningitis, of whom 42 had underlying disease [256]. Moosa and Coovadia reported an in hospital death rate for HIV negative patients of 9% (95%CI 7.1 – 40.6%) [257]. To my knowledge, none of the HIV negative patients in our series had underlying malignancies or were taking immunosuppressive drugs (other than corticosteroids). The death rate in our patients, at the lower end of the spectrum compared with the literature, probably reflects this fact.

3.5.3 Prognostic indicators

Over 40 prognostic indicators have been identified in cryptococcal meningitis. This high number may be a consequence of study methodology, where multiple testing of baseline variables results in associations which are the consequence of chance rather than

genuine. In an attempt to be more rigorous, I elected to test previously defined prognostic indicators on the dataset, rather than to identify prognostic variables from within that dataset. Of all the previously identified prognostic factors, I found that 7 (HIV infection, confusion, Glasgow Coma Score, cerebrospinal fluid cryptococcal antigen titre, cerebrospinal fluid viable yeast count, CSF white cell count and CSF glucose) were associated with death at both 70 days and 6 months, and in addition an abnormal chest X-ray was associated with death at 6 months. When prognostic factors were examined by HIV serostatus, there were some differences, but these must be considered in the context of the reduced sample size. For HIV patients, male gender alone was associated with a reduced hazard for death at both 70 days and 6 months, and focal neurological symptoms were associated with an increased hazard for death at 6 months. Why male gender was protective against death is not clear, but may represent culturally different opportunities for health seeking behaviour in families with limited resources. For example, it is traditional in Viet Nam for married couples to live with the groom's parents, and finances are often controlled by the groom's family, such that wives do not have any financial independence. Since most health care must be paid for, this can impair women's access to care. An alternative explanation is that physiological differences between the sexes somehow affect pathogenesis. However, human sex hormones that have been shown to regulate growth of other fungi *in vitro* (for example 17-beta-oestradiol and progesterone stimulate growth of *Coccidiodes immitis*) have not been found to have the same effect for *C. neoformans* [276, 277].

In HIV uninfected patients, age, confusion, lower Glasgow Coma Score, hemiplegia, blood urea and CSF cryptococcal antigen titre were associated with an increased hazard

of death. The effect of HIV infection presumably obscures the effects of these other factors in the HIV infected group.

The multivariate analysis was carried out only on the 6 month end point and for the group of patients as a whole. This is because there is a danger of 'over-fitting' a model where the model size is small, and where the number of events is relatively low [278]. Since there were more deaths at 6 months compared with 10 weeks, using the 6 month endpoint allowed us to enter more variables into the model, which may contribute important data. Eleven variables met the inclusion criteria for the model which initially included 99 patients. Five variables remained in the final model. Three factors were associated with an increased hazard of death with a significance level of $P<0.05$ – higher log2 CSF cryptococcal antigen titres, lower CSF white cell count and lower Glasgow Coma Score. The other 2 variables left in the model were abnormal chest x-ray ($P= 0.098$) and hemiplegia ($P = 0.093$). There were 104 patients where data for all 5 variables were available, and when the model was refitted on these cases log2 cryptococcal antigen count, Glasgow coma score and log10 CSF white cell count remained significantly associated with survival ($P<0.001$ for all variables). A bootstrap analysis showed that the model selection of these 5 variables was reasonably robust: all selected variables were included in the final model in at least 50% of bootstrap resamples of the data and the variables log2 CSF cryptococcal antigen titre, Glasgow Coma Score and CSF white cell count were selected at least 75% of the time. Interestingly, HIV status was not independently associated with an increased hazard for death in the multivariate model. This is because it is associated with low CSF lymphocyte counts. When HIV status was forced into the model, the same 3 variables

were independently associated with increased hazard for death. It should be noted that the p-values generated by the model should be interpreted with caution – they are biased low since they do not account for the fact that the model is the result of a selection process [279].

If the study size were large enough, and there were enough deaths, all the identified risk factors could have been included in the final model. Given that the number of events (deaths) should be at least 10 times the number of variables tested, this model would have required at least 1300 patients [278].

Of the factors identified by multivariate analysis, the Glasgow Coma Score and log2 cryptococcal antigen titre were also associated with an increased hazard of death by univariate analysis, both for the cohort as a whole and for HIV uninfected patients at 70 days and 6 months. For HIV patients, there was a suggestion that Glasgow Coma Score at presentation may have prognostic value for 10 week survival, but the values did not reach conventional levels of statistical significance ($p = 0.07$). I may have failed to show an association for cryptococcal antigen titre and outcome for HIV infected patients alone because the fungal burden in general was so high in the HIV cohort – median antigen titre was 8-fold higher than in HIV uninfected patients.

The factors identified by the final model as having prognostic significance are biologically plausible, and each was previously identified as a prognostic factor by more than one study (table 3.8).

3.6 Conclusions

Published reports on cryptococcal meningitis are from highly heterogeneous populations, both HIV infected and uninfected, rendering the effect of HIV on the clinical phenotype difficult to determine. Underlying disease is rare in HIV uninfected patients with cryptococcal meningitis in Viet Nam, allowing a clearer understanding of the impact of HIV on disease. I found Vietnamese patients to have differences in age and gender by HIV serostatus – differences that are explained by the demographics of the HIV epidemic in Viet Nam. Of note, I found the duration of illness in HIV infected patients to be shorter than in HIV uninfected patients, and headaches to be a more prominent symptom. The rate of visual impairment is similar between groups and high at over 30%. Differences by HIV serostatus are more marked on clinical investigation, with little evidence of inflammation in HIV infected patients. The fungal burden in HIV patients is considerably higher, as is the risk of death. Most disease in Viet Nam is due to infection with *C. grubii*, irrespective of HIV serostatus, and underlying potentially predisposing disease is found in only 20% of HIV uninfected patients. CSF cryptococcal antigen titre, CSF white cell count, and Glasgow Coma Score are independently correlated with an increased hazard of death at 6 months.

Chapter 4

The Molecular Epidemiology of Human Isolates of *Cryptococcus* Species in Viet Nam.

4.1 Introduction

The aim of the work described in this chapter of the thesis was to determine the molecular epidemiological relationship between strains of *Cryptococcus neoformans* causing meningitis in HIV infected and uninfected patients, the underlying hypothesis being that the strains infecting HIV negative patients had relatively increased virulence, and formed a distinct population. To achieve this I first used Polymerase Chain Reaction – Restriction Fragment Length Polymorphism analysis (PCR-RFLP) of the *URA5* (orotidine monophosphate pyrophosphorylase) gene to determine the infecting species and molecular group [35]. To further define the strain relationships I used a high resolution whole genome-based molecular typing method – Amplified Fragment Length Polymorphism analysis (AFLP) [34, 280, 281].

4.2 Population structure of *Cryptococcus neoformans*

Three different varieties of the encapsulated basidiomycetous yeast *Cryptococcus neoformans* exist [36, 248]. *Cryptococcus neoformans* var *grubii* occurs world-wide, is found associated with pigeon guano in the environment and predominantly causes disease in the immunocompromised [282, 283]. *Cryptococcus neoformans* var *neoformans* occurs mainly in Western Europe, and again tends to affect immunosuppressed patients [270]. While infection in almost all body tissues has been

described, the most common site of disease is the central nervous system. *Cryptococcus neoformans* var *gattii* (*Cryptococcus gattii*) occurs mainly in the tropics and sub-tropics, although since 1999 disease has been reported from British Columbia, Canada and there is now good evidence of autocthonous spread [63, 69, 284, 285]. Most cases of *C. gattii* are reported from Papua New Guinea and Australia where it is associated in the environment with eucalyptus trees [48, 60, 269, 286, 287]. *C. gattii* usually affects immunocompetent patients and the commonest disease presentations are lung disease or meningoencephalitis [76, 269, 288-293]. Separate species status has been proposed for *C. gattii* [21]. It can be distinguished from *Cryptococcus neoformans* var *grubii* and *Cryptococcus neoformans* var *neoformans* using Canavanine-Glycine bromothymol blue agar (CGB agar). *C. gattii* is inherently resistant to the antifungal L-canavanine contained in the medium [294]. The agar also contains a colour indicator - bromothymol blue. Yeast growth causes a change in pH with a resultant change in agar colour from yellow to blue. There is a single report in the literature of a canavanine resistant *C. neoformans* var *grubii* strain[295].

The 3 varieties are also serologically distinguishable. Evans and Kessel showed in the 1940s that rabbit sera could be used to define 3 serotypes A, B and C[16-18]. Subsequently a 4th serotype D was identified, and a hybrid AD strain discovered in the 1980s[19, 20]. In general, *Cryptococcus neoformans* var *grubii* types as serotype A, *Cryptococcus gattii* as serotype B or C, and *Cryptococcus neoformans* var *neoformans* as serotype D, although there are occasional inconsistencies reported in the literature. Worldwide, the vast majority of cases of cryptococcal meningitis occurs in HIV infected patients and are due to infection with *C. neoformans* var *grubii*. In Europe, *C.*

neoformans var *neoformans* accounts for about 25% of cases of cryptococcal meningitis in HIV patients; the rest are due to infection with *C. neoformans* var *grubii* [156]. In Australia and New Zealand Chen at al report that *C. gattii* accounts for less than 0.8% of cases of disease in HIV infected patients, and that 56% of all cryptococcal disease in immunocompetent patients is caused by either var *grubii* or var *neoformans*, although the clinical phenotypes (i.e. whether this is meningitis, pulmonary or other disease) are not described [64]. Overall, 20% of *C. neoformans* var *neoformans* disease was reported to occur in immunocompetent patients.

Few data exist from India, but the dominant isolate is *C. neoformans* var *grubii*, although *C. neoformans* var *neoformans* has been reported [296]. A 3 year surveillance of cryptococcosis from South Africa (1912 isolates) found no serotype D or AD isolates, and that *C. neoformans* var *grubii* accounted for 98% of infections in HIV infected patients, var *gattii* accounting for the remainder. *C. gattii* accounted for 25% of infection in HIV uninfected patients, but there were only 4 such cases[70].

Meyer reported that of 340 environmental, human and animal isolates of *C. neoformans* from 8 South American countries and Spain, 20.4% were *C. gattii*, 73.8% were *C. neoformans* var *grubii* , 0.3% were *C. neoformans* var *neoformans* and 3.8% were AD hybrids[35]. Using *URA5* PR-RFLP, they found that of the *C. neoformans* var *grubii* strains, 92.4% were molecular group VNI, and 7.7% were VNII. Of cerebrospinal fluid derived isolates from HIV positive patients, 87.1% were *C. neoformans* var *grubii* and 3.6% were *C. gattii*. Of CSF-derived isolates from patients who were HIV negative or HIV status unknown, 53.5% were *C. gattii* and 46.5% were *C. neoformans* var *grubii*. In 34 patients with CNS disease in whom there was no underlying disease 61.8% of

infections were due to *C. gattii* and 38.2% due to *C. neoformans* var *grubii* [35]. Of note, there appears to be markedly more intra-species diversity in isolates from North America compared with Brazil[282].

Chen et al determined the genotype of 129 human isolates of *Cryptococcus neoformans* from 16 provinces in China collected over the period 1980 to 2006 [273]. Surprisingly, they report that most cryptococcal meningitis in China seems to occur in patients without underlying disease, HIV being associated with only 8.5% of patients and other underlying disease in 20.5% of patients. 120 of the isolates were *Cryptococcus grubii.* Microsatellite and multi locus sequence typing found that the isolates were highly homogeneous and belonged to a cluster that has diverged phylogenetically from the *URA5* VNI reference strain. There are no data on the molecular epidemiology of *Cryptococcus neoformans* from Viet Nam.

4.3 Molecular typing techniques in cryptococcal research

Numerous molecular typing techniques have been used to investigate the diversity of *C. neoformans*, including karyotyping, RAPD, PCR-RFLP, mini and microsatellite and AFLP typing[34, 35, 246, 282, 283, 297-304]. More recently the consensus loci and primers for multi locus sequence typing (MLST) have been published[305]. PCR-RFLP of the *URA5* gene is a well established and frequently used methodology that divides the *C. neoformans* complex into 8 reproducible molecular groups that correspond with serotyping (see table 4.1) [35].

Amplified fragment length polymorphism analysis is a typing tool that has been used to investigate the genetic diversity of *C. neoformans* both globally, within defined

geographic areas and within micro-biospheres such as single trees [34, 246, 306, 307]. Originally developed as a typing tool for plants, AFLP is popular because it has high resolution, is whole genome based, and is compatible with automated sequencers[280].

Table 4.1

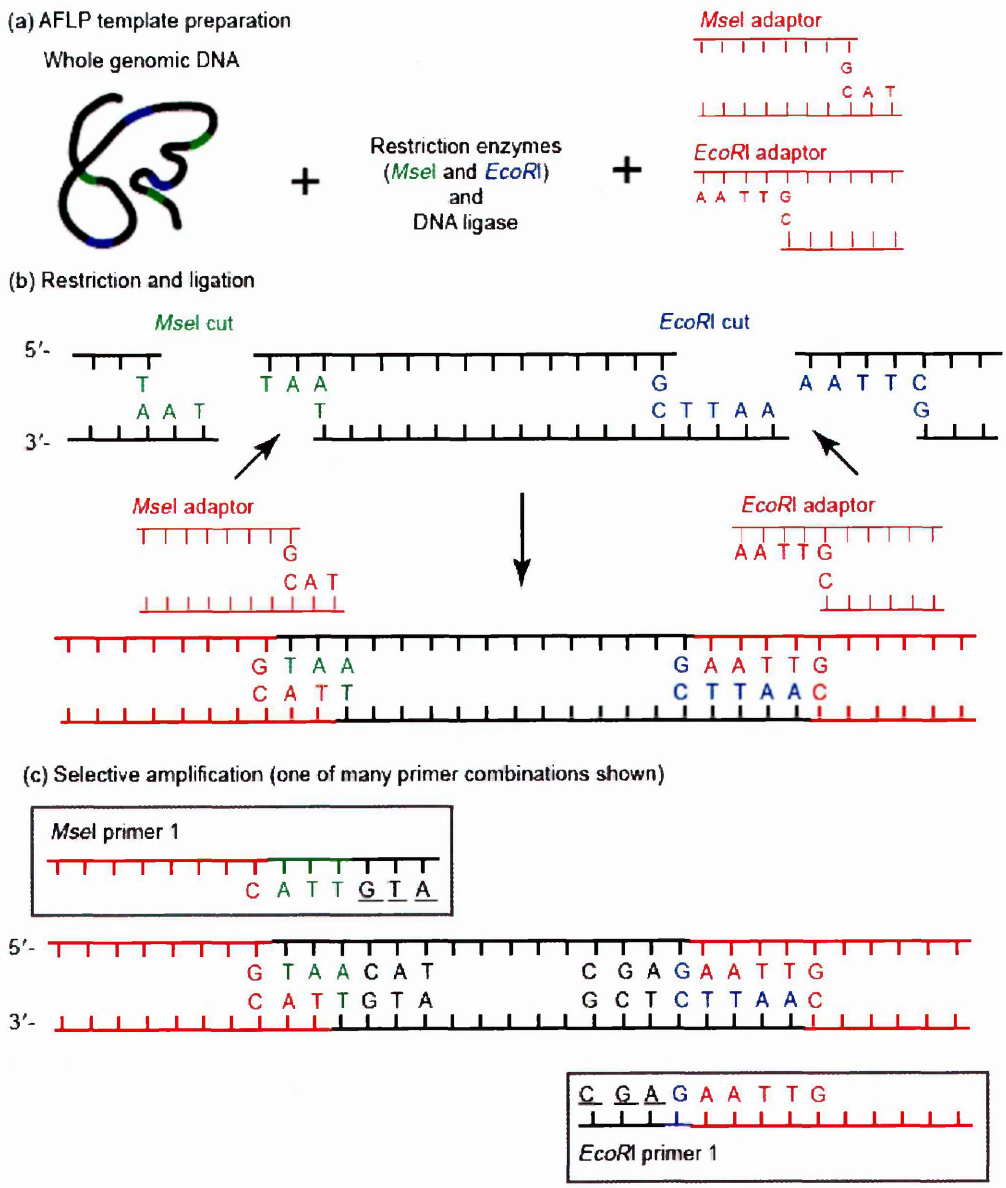
Relationship between species, serotype and *URA5* PCR-RFLP derived molecular group

Species/Variety	Serotype	Molecular Group
Cryptococcus neoformans var *grubii*	A	VNI
		VNII
Cryptococcus neoformans Hybrid	AD	VNIII
Cryptococcus neoformans var *neoformans*	D	VNIV
Cryptococcus gattii	B	VGI
		VGII
		VGIII
	C	VGIV

The process involves extraction of high quality DNA, complete digestion with a frequent and a moderately rarely cutting restriction enzyme, ligation of adapters of known sequence and subsequent rounds of amplification using primers complimentary to the adaptor sequences, which can be made more selective through the addition of further 3' nucleotides to the primer sequence (Figure 4.1). Disadvantages are that it is relatively labour intense, requires high quality (although only small amounts) of genomic DNA, and the multiple reaction stages complicate the optimization process. The first use of

AFLP to type *Cryptococcus* spp was published by Boekhout in 2001[34]. He described 6 major molecular types defined by AFLP. He used primers complimentary for the adapter sequence and made further specific by adding 5'- AC-3' to the EcoR1 primer and 5'-G-3' to the Mse1 adapter. Other groups have used multiple primer sets with greater selectivity[34, 246, 307]. Unfortunately most researchers make no mention of how reproducible their results are when a single isolate is repeatedly typed, so the amount of difference between strains that is due to experimental error rather than genuine variation is not clear. However, most groups define clades at the level of 85% similarity [34, 307].

Figure 4.1 Schematic representation of AFLP. Adapted from Mueller and Wolfenbarger [308].



4.4 Methods

4.4.1 Strains

Strains from HIV uninfected patients were sequentially collected isolates from patients enrolled into the BMD study (see the methods section) collected between 1996 and April 2009. Strains from HIV positive patients were selected randomly from sequentially collected isolates from patients enrolled into the BK study (described in the methods section) between April 2004 and April 2009. All strains were confirmed as *Cryptococcus* species using classical mycological methods as described in chapter 2, including colony morphology, microscopy, characteristic growth on bird seed agar plates, urease production, sugar assimilation tests and biotyping with canavanine-glycine bromothymol blue agar. Control strains used for AFLP and RFLP were the 8 *URA5* PCR-RFLP defined molecular types provided by Associate Professor Wieland Meyer, Westmead Millennium Institute for Medical Research, Sydney, Australia.

4.4.2 DNA extraction

Single colonies of each isolate were spread for confluent growth on Sabouraud's agar and incubated at 30*C.* After 24 hours incubation plates were scraped for DNA extraction using the modified Wen protocol as detailed in Chapter 2.

4.4.3 RFLP

The *URA5* gene was amplified and digested according to the method of Meyer et al, as detailed in Chapter 2 [35]. Product was run on a 3% agarose gel at 100V for 3 hours, the products stained with ethidium bromide and the banding patterns visually inspected and compared with the 8 control strains to determine the *URA5* PCR-RFLP molecular type.

4.4.4 AFLP

The final protocol was derived from the protocol of Associate Professor Dee Carter's laboratory, Department of Microbiology, University of Sydney, and has been described in the Chapter 2 [246]. The optimization process is described in the following results section. Two primer sets were used to generate the genotypes, one set with slightly greater selectivity than the other. The primers sets are detailed in the methods chapter (Table 2.3). Fragment analysis was performed on an AB systems 3130X sequencer (AB Biosystems Inc, Foster City, CA) with 50cm capillaries according to the manufacturer's instructions. A LIZ500 internal standard (35 – 500 base pairs) was included with each experiment. Trace files were generated using the AFLP protocol of the Genemapper version 4.0 (AB Biosystems Inc, Foster City, CA) software package. Raw file data were imported directly into Bionumerics version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium) for analysis.

AFLP data for individual experiments were compared in two ways. First, the densitometric curves for each isolate were compared using the Pearson correlation. Only the curves within the range of the internal size standard (35 – 500 base pairs) were used in the analysis. I tested AFLP consistency through duplicate experiments on single

control isolates. For this analysis, trees were constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). This algorithm uses the highest average similarity between two entries or clusters to determine which ones to merge, and provides an easy measure of the per cent similarity between 2 isolates. However, UPGMA does not perform as well where distances between isolates are longer, and assumes a constant rate of evolution [309]. Therefore, for the definitive analyses, where the actual relationship between isolates was not known, I used the Neighbour Joining Tree method (NJT) [310]. The NJT method has been shown to be statistically consistent under many models of evolution, and performs favourably compared with other tree construction methods[311, 312]. The algorithm uses the lowest sum of all branch lengths as it merges entries and clusters, starting with a "star tree" of equal branch lengths and ending with an unrooted dendrogram. It differs from other algorithms in that it does not assume that the rate of evolution remains constant throughout the dendrogram. The statistical significance of the clusters was tested by cophenetic correlation. The advantage of using densitometric curves for analysis is that all the information between defined gel distances (band sizes) is used, protecting the procedure from operator bias and loss of potentially informative bands. However, it is not possible to perform a bootstrap analysis.

Secondly, I performed a band-based analysis. I used the automated band assigning algorithm within Bionumerics 5.1 to define polymorphic band classes across the AFLP dataset, and these were used to generate NJTs for each primer set. Only band classes that were intense, polymorphic and that occurred within the range defined by the internal size standard were used in the analysis. The Dice method was used to create the trees,

and the statistical significance of the clusters was tested by cophenetic correlation and bootstrap analysis. The disadvantages of using a band-based approach for analysis are that it is open to investigator bias, and potentially informative information is lost through the choice of rules to define bands. However, bootstrap analysis can be performed. Experiments, from DNA extraction to final amplification, were performed on batches of 24 strains. The strains for each batch were randomly selected using a random number sequence generated in an Excel spreadsheet (Microsoft Office 2003, Microsoft, Seattle, USA). In the VN1 strain analysis 192 experiments, representing each HIV negative strain in duplicate, 2 *C. neoformans* var *grubii* and 2 *C. neoformans* control strains in duplicate, and 96 HIV associated strains were fragment analysed in a single run and uploaded simultaneously to Bionumerics v5.1 (Applied Maths NV, Sint-Martens-Latem, Belgium) for normalisation and analysis. Fishers' exact test was used to compare the proportions of strains in identified clades by the HIV infection status of the isolate source.

4.5 Experiment optimization

The experimental optimisation process will be described first, followed by the experimental results.

4.5.1 DNA Extraction

Whole genomic DNA was obtained using the modified Wen protocol [245]. All extracts were treated with RNAse. The median concentration of extracted DNA was 1143.58 ng/uL (range 105.81 ng/uL to 4992.4 ng/uL).

4.5.2 RFLP Optimization

URA5 PCR and restriction digestion worked without protocol modification. All 8 control strains were easily distinguishable (see figure 4.2).

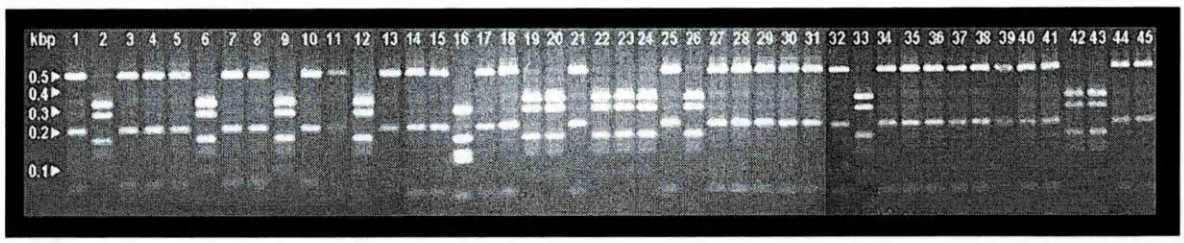


Figure 4.2 Representative PCR-RFLP profiles of the URA5 gene of 45 *Cryptococcus* isolates. Typical patterns: Lane 1 = VN1, Lane 6 = VG1, Lane 16 = VG2.

4.5.3 AFLP Optimization

Optimization was initially performed using each primer set according to the unmodified Carter protocol. Using 200 to 600ng of genomic DNA and the unmodified protocol resulted in poor quality bands, despite digestion appearing to have been complete. A systematic approach was adopted in order to problem solve the technique and optimise conditions. All optimization experiments were performed on genomic DNA from the same 4 isolates, using the Carter lab pre-amplification and selective amplification PCR settings, and the Boekhout lab pre-amplification and selective amplification PCR settings[34, 246]. Electrophoresis of product on agarose gels and visual inspection for bands was used to determine the impact of protocol changes. DNA digestion and ligation were carried out in 0.2ml PCR tubes. Digestion was carried out at 37C in a water bath. Ligation was performed at 22C in a PCR machine (BioRad Tetrad2 DNA Engine, BioRad Laboratories Ltd, Hemel Hempstead, UK). Polymerase chain reaction amplification was performed using a BioRad Tetrad2 DNA Engine (BioRad Laboratories Ltd, Hemel Hempstead, UK).

4.5.3.1 Optimization Experiment 1:

Hypothesis: Restriction digestion is incomplete

A digestion check control is built into the protocol using lambda DNA. However, failure of one enzyme to cut efficiently would not be detected. Failure of one enzyme to cut would result in failure of ligation and subsequent amplification. In order to confirm that both enzymes EcoR1 and MseI were digesting efficiently each was incubated separately with lambda DNA and the standard reaction conditions.

Results: Cleavage occurred with each restriction enzyme (Figure 4.3). EcoR1 digestion produced the expected 6 bands [313]. The more frequently cutting Mse1 produced the expected restriction smear.

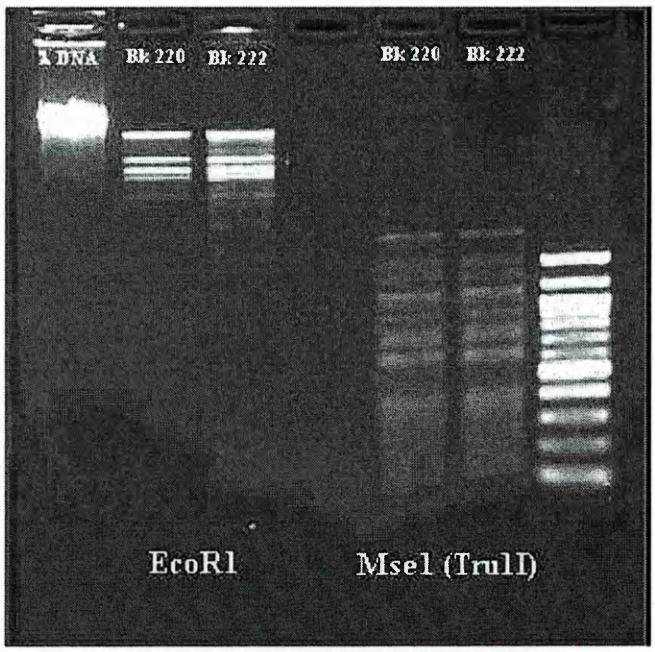


Figure 4.3 Separate restriction digest of lambda DNA with EcoR1 and Mse1.

Conclusion: Both enzymes cut efficiently in the protocol digestion buffer.

4.5.3.2 Optimization experiment 2:

Hypothesis: amplification is inefficient due to inadequate Magnesium Chloride or *Taq* DNA polymerase concentrations.

I tested the effects of adding additional $MgCl_2$ and altering the amount of *Taq* polymerase in the reaction mixes. These 2 effects were tested in a factorial design (see table 4.2).

Table 4.2

Optimisation experiment 2 design

Total reaction volume 25uL	**0.075 uL Magnesium Chloride 50mM**	**0.75 uL Magnesium Chloride 50mM**
0.5 uL *Taq* DNA Polymerase 5U/uL	A	B
1 uL *Taq* DNA Polymerase 5U/uL	C	D

Results: Bands were generated only with the higher concentration of magnesium chloride, and were better quality with the higher Taq polymerase concentration (figure 4.4).

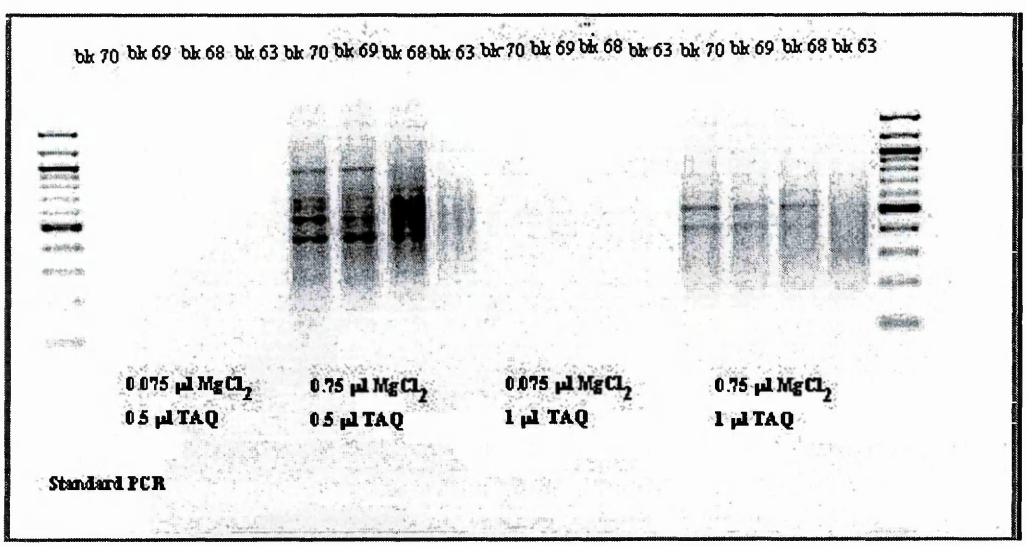


Figure 4.4. Effects of varying magnesium and Taq concentrations on band amplification.

Conclusion: Amplification was more efficient with 0.75uL of 50mM magnesium chloride and 5U (0.5 uL) of Taq polymerase per reaction. However, overall the quality of the bands was poor.

4.5.3.3 Optimization Experiment 3

Hypothesis: Either the pre-selective or selective primers were failing to generate product

In order to determine whether there was a problem with either the pre-selective or selective primer set, the protocol was run using

i) only the pre-selective primers in both the selective and pre-selective amplification steps,

ii) only the selective primers in the pre-selective and selective amplification steps, and

iii) using the selective PCR primers on the product from experiment i).

In addition, the normal protocol was run as a control. All reactions used 0.75uL of $MgCl_2$ 50mM and 5U Taq polymerase.

Results: Amplification occurred with each individual primer set, but bands remained faint (figure 4.5).

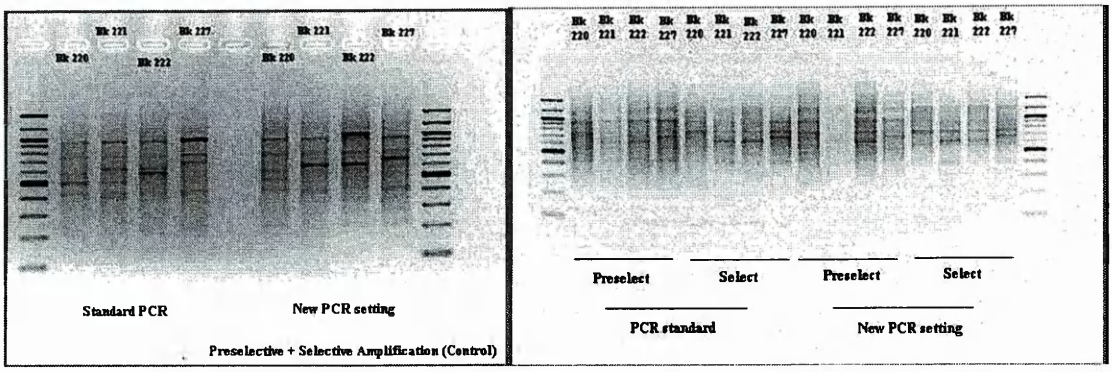


Figure 4.5 The effect on amplification of using preselective primers for both rounds of amplification (upper gel, labelled 'preselective'), selective primers for both rounds of amplification (right gel, labelled 'selective'), and the standard protocol (left gel).

Conclusion: Amplification is possible with each primer set.

4.5.3.4 Optimization experiment 4

Hypothesis: There was too much substrate (DNA) for effective ligation/amplification

Efficient and reproducible AFLP depends upon there being reagent in excess, to ensure that there is complete digestion, ligation and amplification. The DNA concentration used in the Carter protocol is 20 fold higher than in the Boekhout protocol (200ng versus 10 ng), although the primers used by each group differed in their selectivity. I considered that the poor band production may have been related to too many annealing sites for too low a concentration of primer, or insufficient other reagents at any of the particular stages (digestion, ligation or amplification). Thus I next experimented with the DNA concentration (Table 4.3).

Table 4.3

Optimization experiment 4 design	
Experiment	**DNA added (ng)**
1	200 – 600
2	40-120
3	20-60
4	2-6

Results:

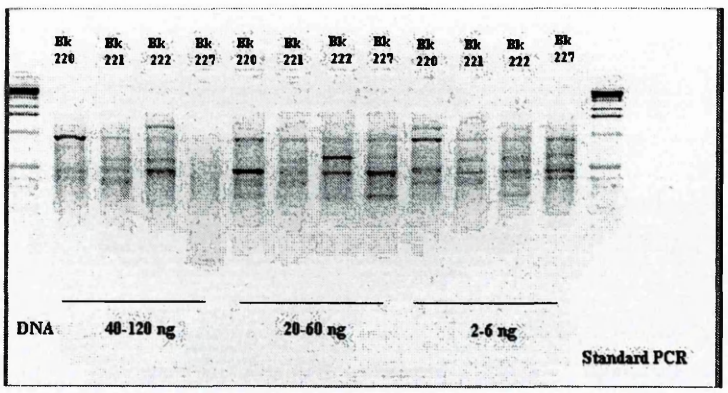


Figure 4.6.The effect of altering the quantity of DNA on band generation.

The clearest bands were produced with 40ng of DNA, and reasonable bands were produced with 2 – 6 ng (Figure 4.6).

Conclusion: 40ng of DNA seemed to result in most efficient amplification.

4.5.3.5 Optimization Experiment 5

Hypothesis: poor band amplification was due to inadequate primer concentrations

The stock primer solution concentration was 25uM. I experimented with primer concentration in the reaction mix: Initially the concentration of pre-selective primer was varied – 1, 2 or 4 uL were added to the final reaction mix of total volume 25uL. In each

reaction product 3 different concentrations of selective primer were used: 0.5, 1.0 or 2 uL of primer solution were added.

Results:

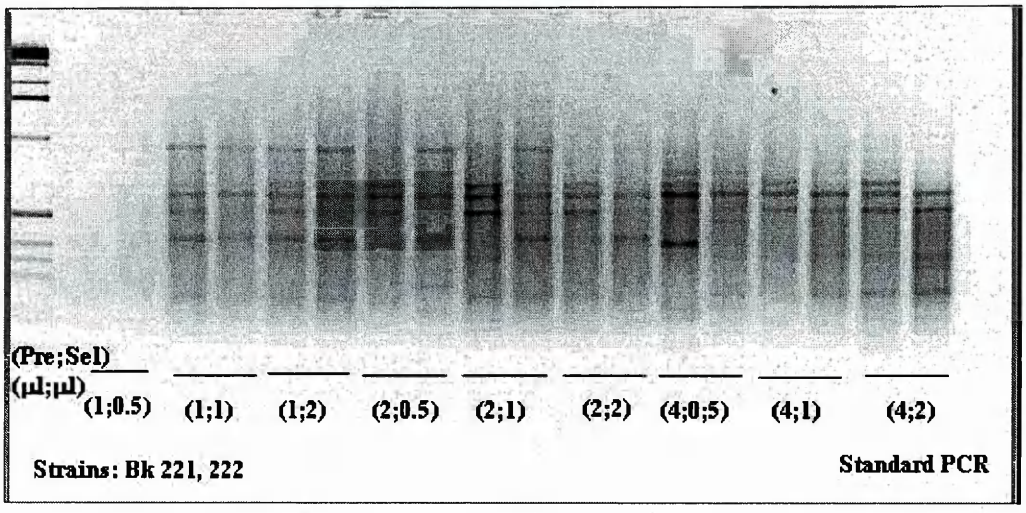


Figure 4.7 The effect of altering primer concentration in preselective (1, 2 or 4 uL per reaction) and selective (0.5, 1 and 2 uL per reaction) reactions on band amplification.

Using 2uL of pre-selective primer and 0.5uL of selective primer gave the cleanest and best intensity bands (figure 4.7). However, this experiment was not reproducible. The primer stock was old - the experiment was repeated with new primer sets.

4.5.3.6 Optimization Experiment 6

Hypothesis: Variability in amplification efficacy was due to old primers.

DNA was re-extracted from the test strains, digestion and ligation performed according to the optimized protocol using 20 to 60ng of DNA. New primer stocks were used. 2uL of pre-selective primer was used and product was amplified with 0.5, 1 or 2uL of selective primer.

Results: Good bands were achieved with the GT primers and 0.5uL of selective primer, but the AC bands were faint - there appeared to be some bands present following pre-selective amplification, but after selective amplification bands were present but very sparse. It was considered that a trial of a different PCR machine might be helpful.

4.5.3.7 Optimization Experiment 7:

Hypothesis: Inconsistent band amplification was due to PCR machine variability

The experiment was repeated with 2uL of pre-selective primer and 0.5 uL of selective primer and using the BioRad C1000 thermal cycler (BioRad, Hemel Hempstead, UK) in place of the BioRad Tetrad2 DNA Engine.

Results: Figure 4.8 shows products generated with 2 different PCR machines. Band quality was improved (Figure 4.8)

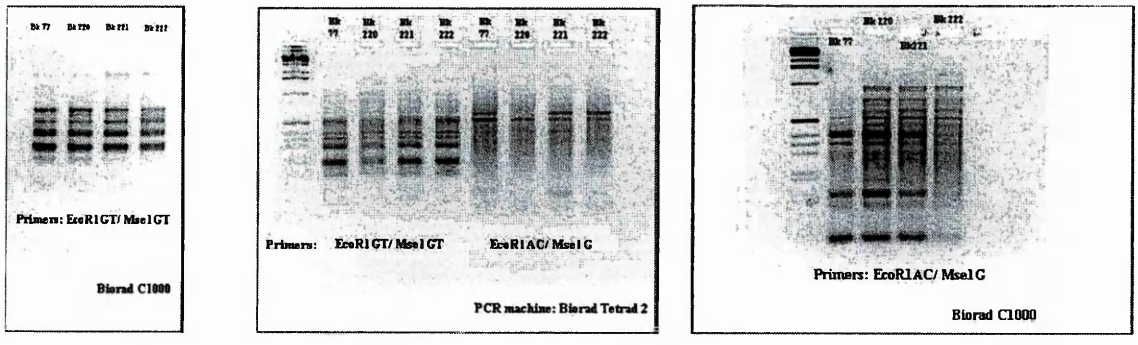


Figure 4.8.Comparison of the BioRad Tetrad 2 PCR engine (centre gel) with the BioRad C1000 thermal cycler (left and right gels) on AFLP amplification efficiency. 110V, 90mins.

Conclusion: sharper more intense bands were generated with the BioRad C1000 thermal cycler.

4.5.3.8 Optimization Experiment 8

Aim: determination of optimal product dilution for fragment analysis

An AB Biosystems 3130x Selective amplification product was diluted 5 fold, 10 fold, 50 fold, 100 fold and 500 fold and run on the sequencer according to the manufacturer's instructions.

Results: The 1:10 dilution gave good quality peaks without clipping (Figures 4.9a and 4.9b)

Figure 4.9.GT primer set AFLP trace file – a) *C. neoformans* var *grubii* VN1. b) *C. gattii* VG1 control strains (Genemapper version 4.0). Blue peaks represent isolate fragments, gold peaks the LIZ 500 internal size standard.

Fig 4.9a)

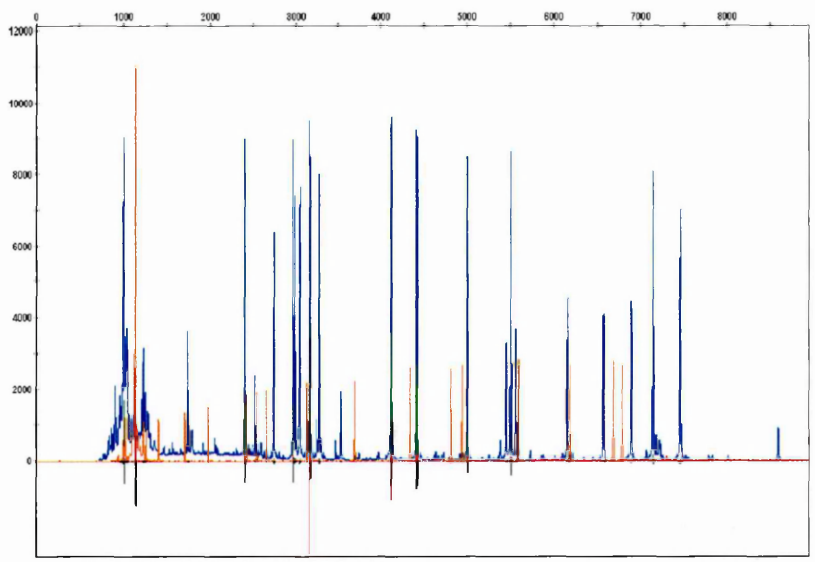
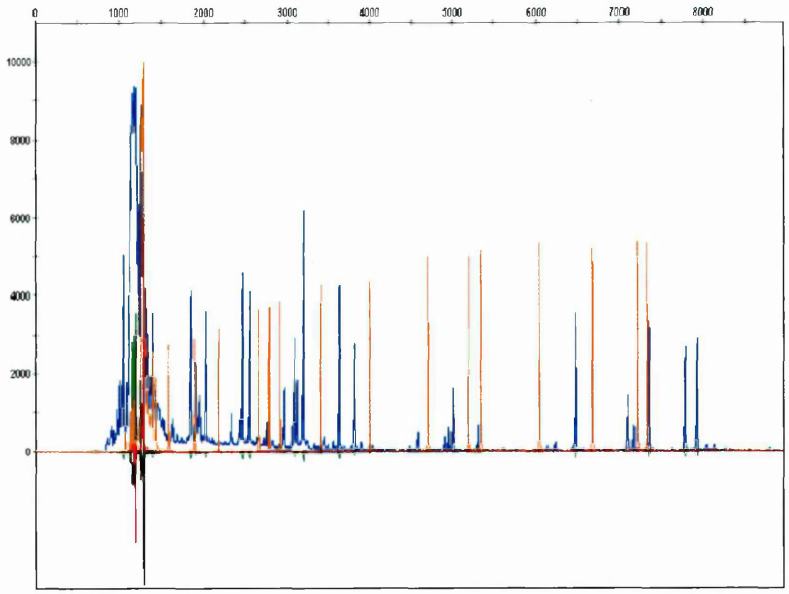


Fig 4.9b)



4.5.3.9 Optimization Experiment 9

Aim: AFLP of all 8 control strains and patient isolates with GT primer pair

AFLP was carried out with the above modifications.

Results: Excellent band patterns on agarose gel (figure 4.10).

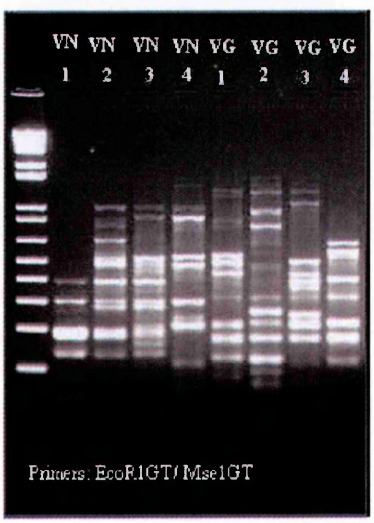


Figure 4.10 AFLP band pattern with GT/GT primers for the 8 control strains. 110V, 90mins.

The GT primer set was thus optimized. The following optimization experiments relate only to the AC/G primer set.

4.5.3.10 Optimization Experiment 10

AC/G primer pairs would be expected to produce approximately four times as many bands as -GT/-GT primer pairs. I considered whether this explained the decreased intensity of bands seen on the AC/G gels.

Hypothesis: The faint and poorly defined bands seen with -AC/-G primer amplification compared with -GT/-GT amplification are consistent with the increased number of bands generated. Amplification with similarly selective AC-GT primer pairs will produce band patterns similar to the -GT/-GT primer pairs.

I performed selective amplification with -AC/-GT primer pairs to see if this resulted in fewer more intense bands, and a gel pattern similar to that obtained with -GT/-GT primer pairs.

Results: Amplification was successful but the bands produced were still less intense than those produced by the -GT/-GT primer pair.

4.5.3.11 Optimization experiment 11

Hypothesis: there was too much substrate for -AC/-G primer binding to enable efficient amplification.

The pre-selective A/G primer product was diluted prior to AC/G amplification. 5ng of pre-selective amplification product was used for each reaction.

Results: Good bands seen on agarose gel. Good traces obtained on fragment analysis without the need for product dilution.

4.5.3.12 Optimization Experiment 12: Reassessment of magnesium concentration for –AC/-G primers.

5ng of preselecive product was used. The $MgCl_2$ (50mM) volume added to the pre-selective reaction mix was 0.25, 0.5 or 0.75uL. Pre-selective product was diluted 1 in 25 or 1 in 10 for each selective amplification round. Each selective amplification round took place with 0.25, 0.5 or 0.75uL of $MgCl_2$.

Results:

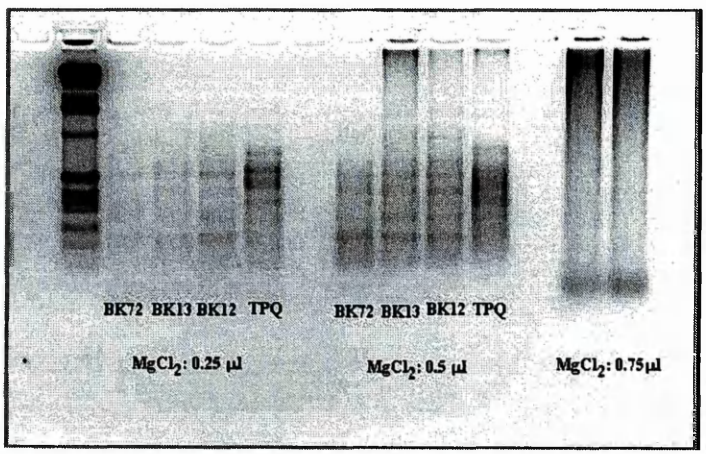


Figure 4.11 The effect of different $MgCl_2$ concentrations on band generation (AC/G primer set). 110V, 90mins.

The best bands were produced when the pre-selective PCR product was diluted 1 in 10 and combined with 0.5uL of $MgCl_2$ (Figure 4.11).

4.5.3.13 Optimization Experiment 13: Increased dNTP concentration

Given that the optimization experiments for the -AC/-G primers suggested that I did not have sufficient reagent in excess (i.e. amplification efficacy improved as the DNA

substrate concentration were reduced) I considered whether increasing dNTP concentrations would improve amplification.

Hypothesis: dNTPs were not present in excess

The concentration of each individual dNTP in the PCR reaction mixes were increased to 20mM from 5 mM and compared with 5mM.

Results: There was no clear difference in band generation by dNTP concentration.

4.5.3.14 Optimization experiment 14

Fragment analysis of AC and GT primer products on the AB 3130x sequencer. Product was analysed with the AFLP protocol according to the manufacturer's instructions. A LIZ 500 base-pair internal size standard was used.

Results: Satisfactory fragment traces were produced with undiluted amplification product.

This completed the -AC/-G primer set optimization process.

Figure 4.12 shows the typical band patterns generated with the AC/G and GT/GT primer sets after protocol optimization.

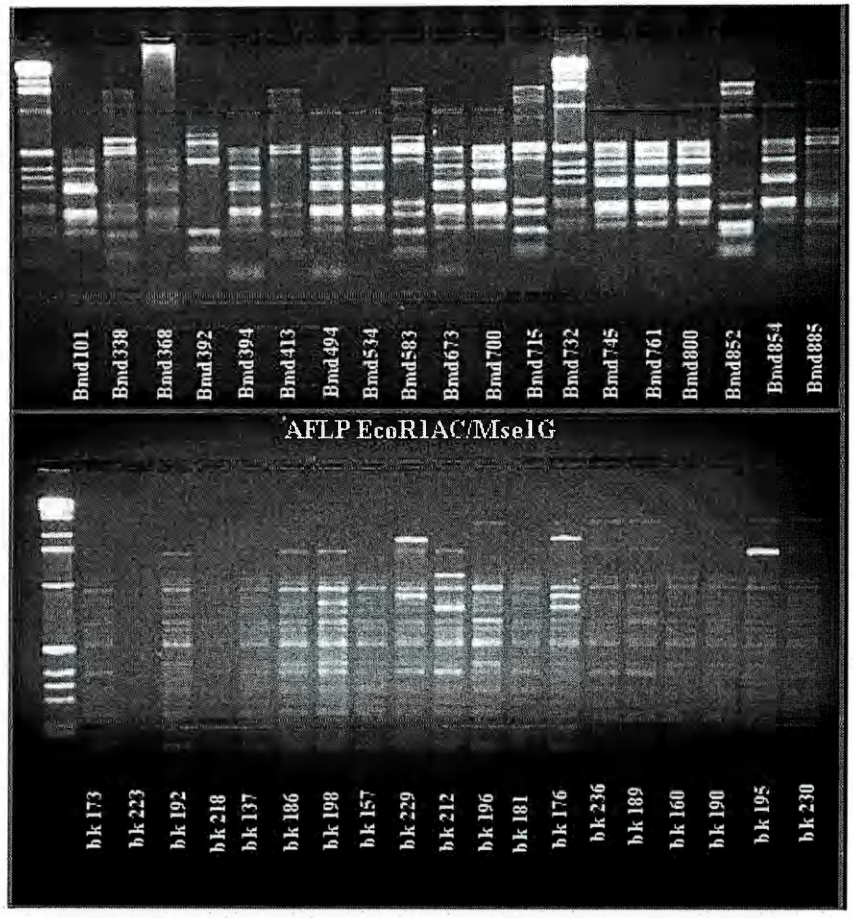


Figure 4.12 Typical band patterns obtained with AC/G primers (top gel) and GT/GT primers (bottom gel), 110V, 100 minutes.

4.5.4 Determination of the reproducibility of AFLP typing

In order to understand the consistency of the typing method, I performed multiple duplicate experiments on the control strains. In my optimized protocol, there were separate digestion and ligation steps. However, since the ligation of adapters does not reproduce the EcoR1 or Mse1 cut sites, it is possible to perform these steps in duplicate. While the optimal temperature for each reaction is different (37C for digestion, 22C for ligation), this is an approach that has been used by some groups [34]. I compared

digestion followed by ligation with simultaneous digestion and ligation overnight at room temperature, and simultaneous digestion and ligation for 2 hours. Each digestion ligation reaction was performed in duplicate, and each digestion ligation product underwent duplicate pre-selective and selective amplification. Each selective amplification product underwent duplicate fragment analysis. Thus a single digestion ligation product resulted in 8 fragment analysis experiments, and for each control strain 24 experiments were performed. Analysis was performed in Bionumerics v5.1. UPGMA trees were generated using the Pearson coefficient to compare the fragment trace files. The percentage similarity between each identical strain was compared according to experiment type. The experimental design is shown in Figure 4.13, and the resulting dendrograms for control strains VN1 (*C. neoformans* var *grubii*), VN4 (*Cryptococcus neoformans* var *neoformans*), VG1 and VG 2 (*Cryptococcus gattii*) are shown in figures 4.14 and 4.15 below.

Figure 4.13 AFLP Consistency experimental design.

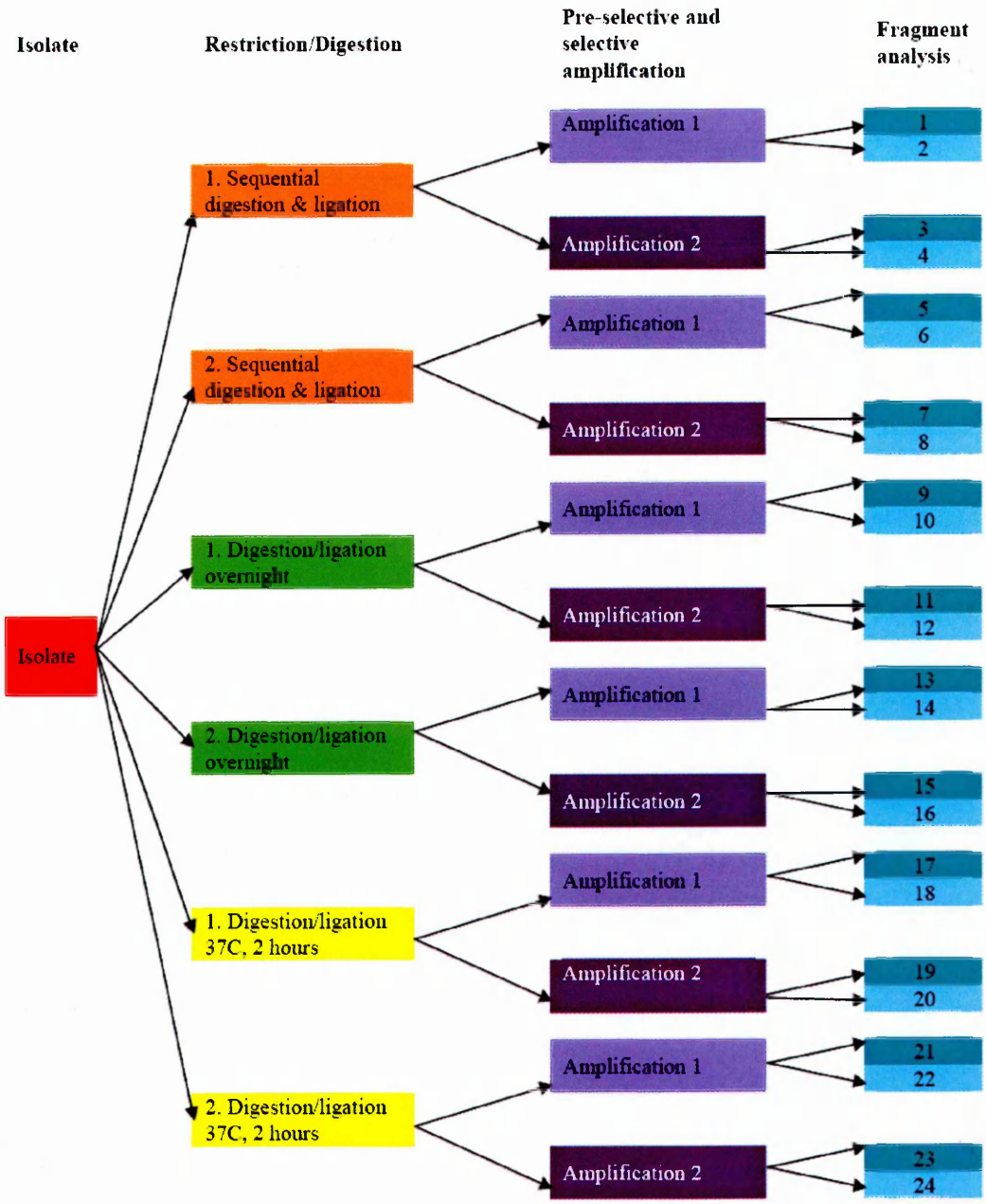
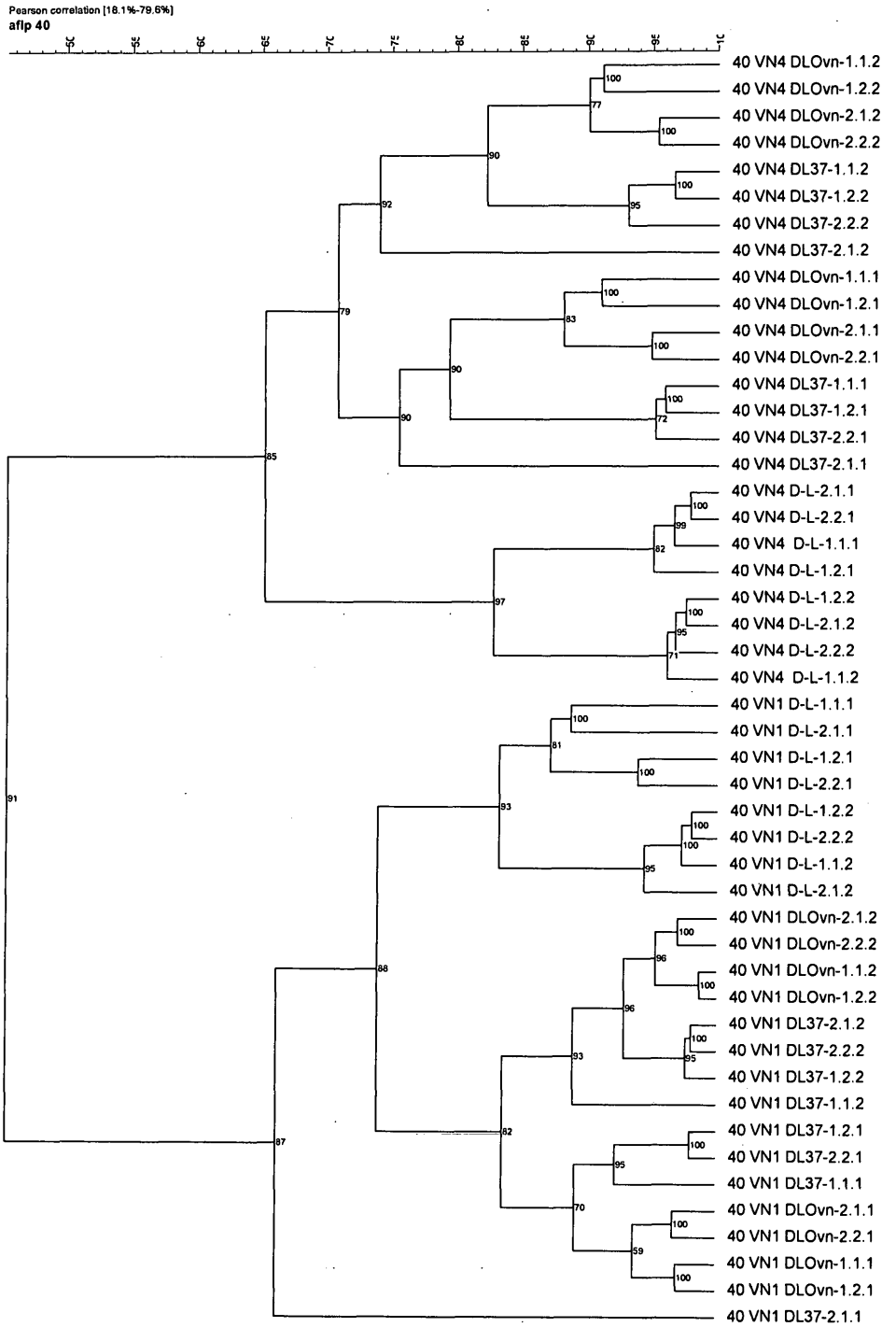
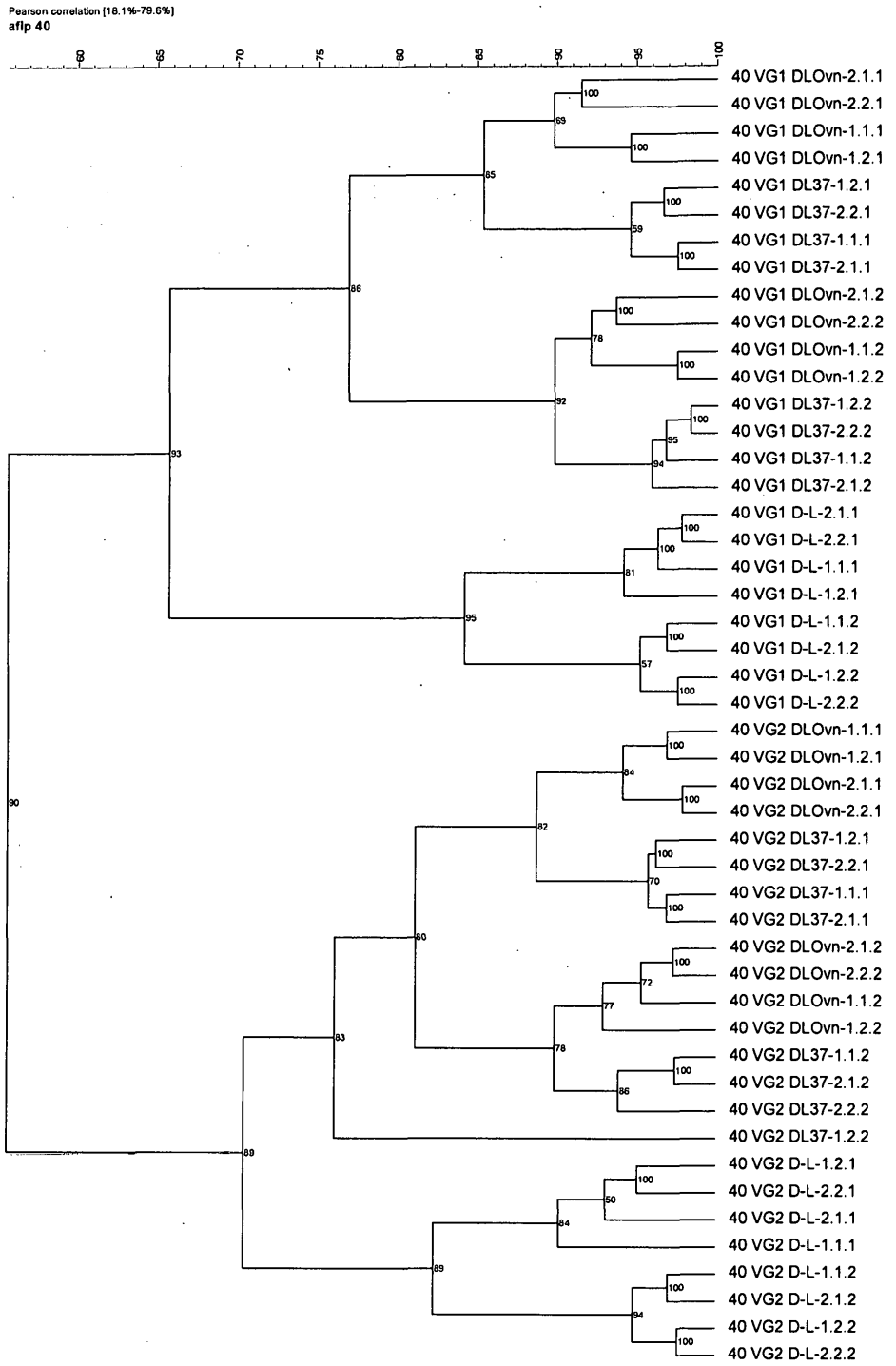


Figure 4.14 AFLP UPGMA dendrogram for *Cryptococcus grubii* (VN1) and *Cryptococcus neoformans* (VN4) control strains.



Nomenclature: VN1/VN4 = control strain; D-L = digestion followed by ligation, DLOvn – simultaneous digestion and ligation overnight at room temperature, DL37 = simultaneous digestion and ligation at 37C for 2 hours ; the first number of the suffix refers to the first or second of the duplicate digestion/ligation experiments, the second number to the first or second round of selective or preselective amplification, and the final number refers to the group (1 or 2) the experiment was in for the fragment analysis run.

Figure 4.15 AFLP UPGMA dendrogram for *Cryptococcus gattii* (VG1) and *Cryptococcus gattii* (VG2) control strains.



Nomenclature: VG1/VG2 = control strain D-L = digestion followed by ligation, DLOvn – simultaneous digestion-ligation overnight at room temperature, DL37 = simultaneous digestion-ligation at 37C for 2 hours; the first number of the suffix refers to the first or second of the duplicate digestion/ligation experiments, the second to the first or second round of selective or preselective amplification, and the final to the group (1 or 2) the experiment was in for the fragment analysis run.

The dendrograms illustrate that the isolates cluster initially by RFLP molecular type – i.e. the AFLP clearly distinguishes the species and the individual isolates. The experiments also cluster by the method used to generate the ligation product, suggesting that this influences the AFLP banding pattern, presumably by generating differing proportions or intensities of substrate DNA fragments. A striking feature of the trees is that clustering occurs by fragment analysis run, and this accounts for the largest variability between experiments for individual isolates. This could be explained by voltage fluctuations during electrophoresis, but could also be explained by contamination during sequencer set-up, or ageing gel or capillaries. Within fragment analysis runs, the reproducibility varied according to the digestion/ligation methodology. The mean similarities between identical isolates according to digestion/ligation methodology are shown in table 4.4 below.

Table 4.4

Per cent similarity values for repeated AFLP experiments on the same 4 control strains according to digestion/ligation method.

Digestion/Ligation Method	Mean similarity	Standard deviation	95% CI	IQR	N
Digestion followed by ligation	94.5	3.05	93.7 – 95.4	94.2 – 96.7	48
Simultaneous digestion/ligation, 37C for 2 hours	89.9	9.62	87.2 – 92.6	86.5 – 96.4	48
Simultaneous digestion overnight	93.2	2.88	92.4 – 94.0	90.8 – 95.3	48

Digestion followed by ligation resulted in the highest similarities for repeated analysis of a single isolate at 94.5%, compared with 93.2% for simultaneous overnight digestion/ligation and 89.9% for simultaneous digestion/ligation at 37°C for 2 hours. Thus the protocol was left unmodified with separate digestion and ligation steps. The variability seen between fragment analysis runs was unexpected, and may have been explained by different gel ages, old capillaries and voltage fluctuations. In order to minimize these effects, the capillaries and gel were replaced, and for the definitive experiments and analysis I decided to fragment analyze the maximum possible number of isolates in a single setting (two 96 well plates, 192 experiments), with the control and HIV negative isolate strains done in duplicate (each represented once on each plate) such that the degree of sequencer introduced error could be estimated.

4.6 Results

4.6.1 Strain characterisation

All strains in the experiment were urease positive. All *C. gattii* strains grew on CGB agar; no *C. neoformans* var *grubii* strains were L-canavanine resistant. Strain BMD 761 (clade 1) was amelanotic, producing cream colonies on birdseed agar. All strains were confirmed as *Cryptococcus neoformans* complex on sugar assimilation testing (Biomerieux API32C, Biomerieux, Craponne, France).

4.6.2 *URA5* PCR-RFLP

53 isolates from HIV uninfected patients enrolled into the BMD study between 1996 and June 2009 were available for analysis. Of these, 39 were *Cryptococcus grubii URA5* PCR-RFLP molecular group VN1, and 14 were *Cryptococcus gattii* (12 *C. gattii* molecular group VG1 and 2 *C. gattii* molecular group VG2. 211 isolates were available from 238 HIV infected patients consecutively enrolled into the BK study. All were *C. neoformans* var *grubii* molecular type VN1. Representative strains and the characteristic banding patterns are shown in Figure 4.2.

4.6.3 AFLP

AFLP using either primer set successfully distinguished *Cryptococcus* isolates by species. Neighbour joining trees for the HIV negative isolates created using the Pearson

product moment correlation comparing densitometric curves are illustrated in figures 4.15 and 4.16 below for both primer sets.

Figure 4.16 Neighbour joining tree showing speciation of Vietnamese *C. neoformans* var *grubii* and *C. gattii* human isolates using AFLP with AC/G primers.

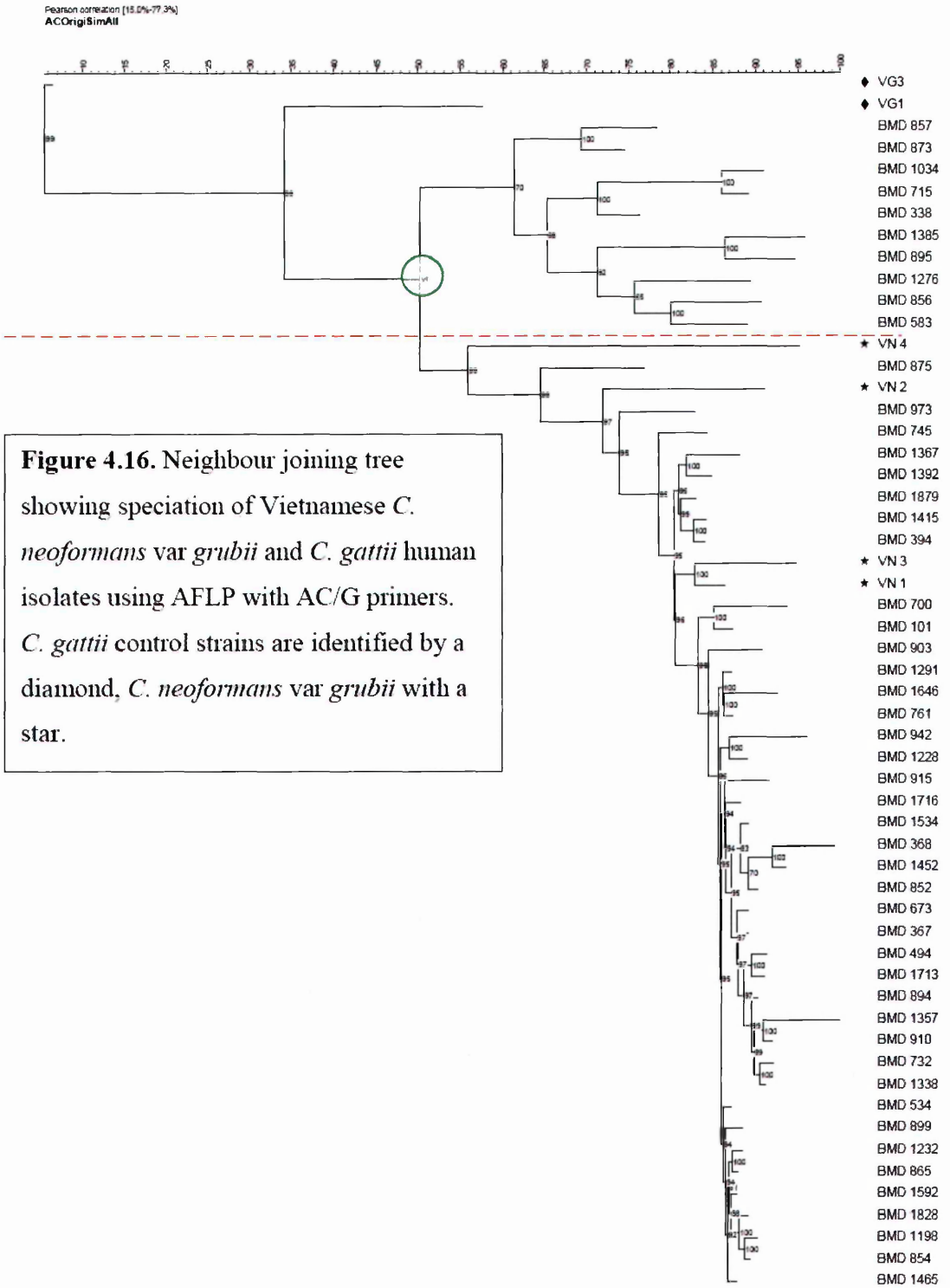


Figure 4.16. Neighbour joining tree showing speciation of Vietnamese *C. neoformans* var *grubii* and *C. gattii* human isolates using AFLP with AC/G primers. *C. gattii* control strains are identified by a diamond, *C. neoformans* var *grubii* with a star.

Figure 4.17 Neighbour joining tree showing speciation of Vietnamese *C. neoformans* var *grubii* and *C. gattii* human isolates using AFLP with GT/GT primers

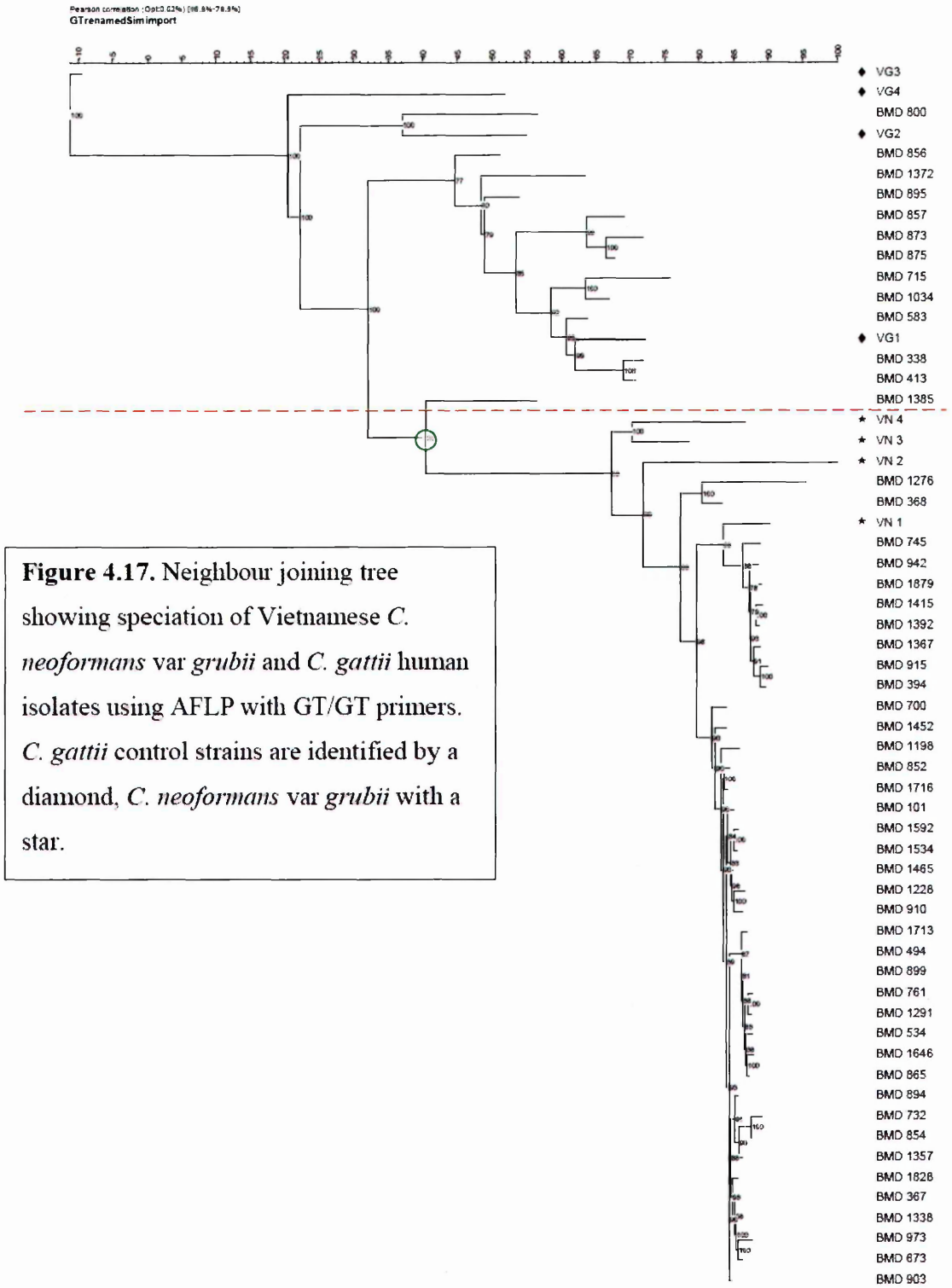


Figure 4.17. Neighbour joining tree showing speciation of Vietnamese *C. neoformans* var *grubii* and *C. gattii* human isolates using AFLP with GT/GT primers. *C. gattii* control strains are identified by a diamond, *C. neoformans* var *grubii* with a star.

Both primer sets distinguished *Cryptococcus gattii* strains from *Cryptococcus neoformans*. The red dotted line divides *C. gattii* (above) from *C. neoformans* var *grubii* strains (below). The nodes at the points of divergence of the species were well supported with cophenetic correlation values of 99 for each primer set (green circles). In the AC/G experiment, one isolate, BMD 875, did not cluster with the other *C. gattii* strains, but appeared more closely related to the VN4 and VN2 control strains. BMD 875 is CGB agar positive, *URA5* PCR-RFLP group VG1, and clusters with the *C. gattii* strains using the GT/GT primer set. Therefore it is almost certainly a *C. gattii* strain. I included this strain in the subsequent experiments investigating the relationships of the *C. neoformans* var *grubii* strains. It consistently clustered with the *C. gattii* out-group with both primer sets (see below). Therefore the failure to cluster with the *C. gattii* in this experiment represents experimental error – most likely in labelling or in fragment analysis.

Thirty-eight isolates from HIV uninfected patients were *C. neoformans* var *grubii* VN1. To determine the relationship of these strains with Vietnamese strains from HIV infected patients, an experiment set was created consisting of these 38, the 2 *C. neoformans* var *grubii* control strains (molecular groups VN1 and VN2), 2 *Cryptococcus neoformans* control strains (VN3 and VN4), and 99 HIV associated strains. In addition, the experiment set contained 3 *Cryptococcus gattii* strains from HIV uninfected patients as an 'out' group. The HIV associated strains were selected randomly from the 238 BK strains using random numbers generated in a Microsoft Excel spreadsheet. The 144 isolates were randomly divided into 6 batches using random numbers generated in Microsoft Excel. AFLP, from DNA extraction up to the final round of amplification was performed by batch. 192 fragment analyses were performed in one run (the maximum

number possible). Fragment analysis was done in duplicate for all HIV negative and control strains - each of the two 96 well plates contained an aliquot from each HIV negative strain and each control. The remainder of the wells were filled with aliquots from the 99 HIV associated strain experiments.

The consistency of the fragment analysis is illustrated in Figures 4.17 and 4.18 which are UPGMA trees generated by comparing densitometric curves using the Pearson correlation for all the duplicate analyses. Table 4.5 shows the mean, standard deviation and percentile similarities between identical isolates for each primer set.

Figure 4.18 AFLP derived UPGMA tree for HIV negative, control and out-group isolate duplicate analyses using the AC/G primer set.

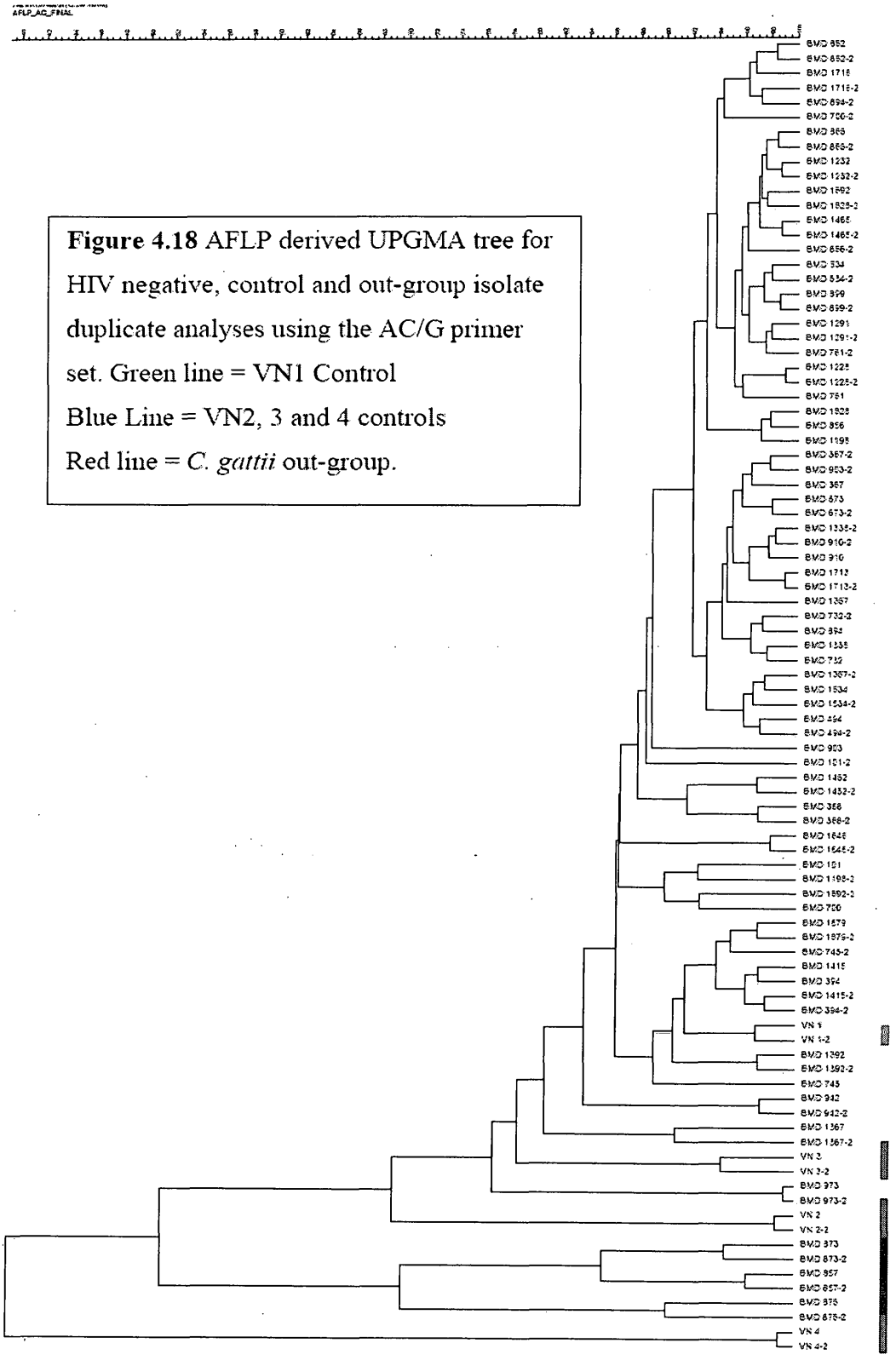


Figure 4.18 AFLP derived UPGMA tree for HIV negative, control and out-group isolate duplicate analyses using the AC/G primer set. Green line = VN1 Control
Blue Line = VN2, 3 and 4 controls
Red line = *C. gattii* out-group.

Figure 4.19 AFLP derived UPGMA tree for HIV negative, control and out-group isolate duplicate analyses using the GT/GT primer set.

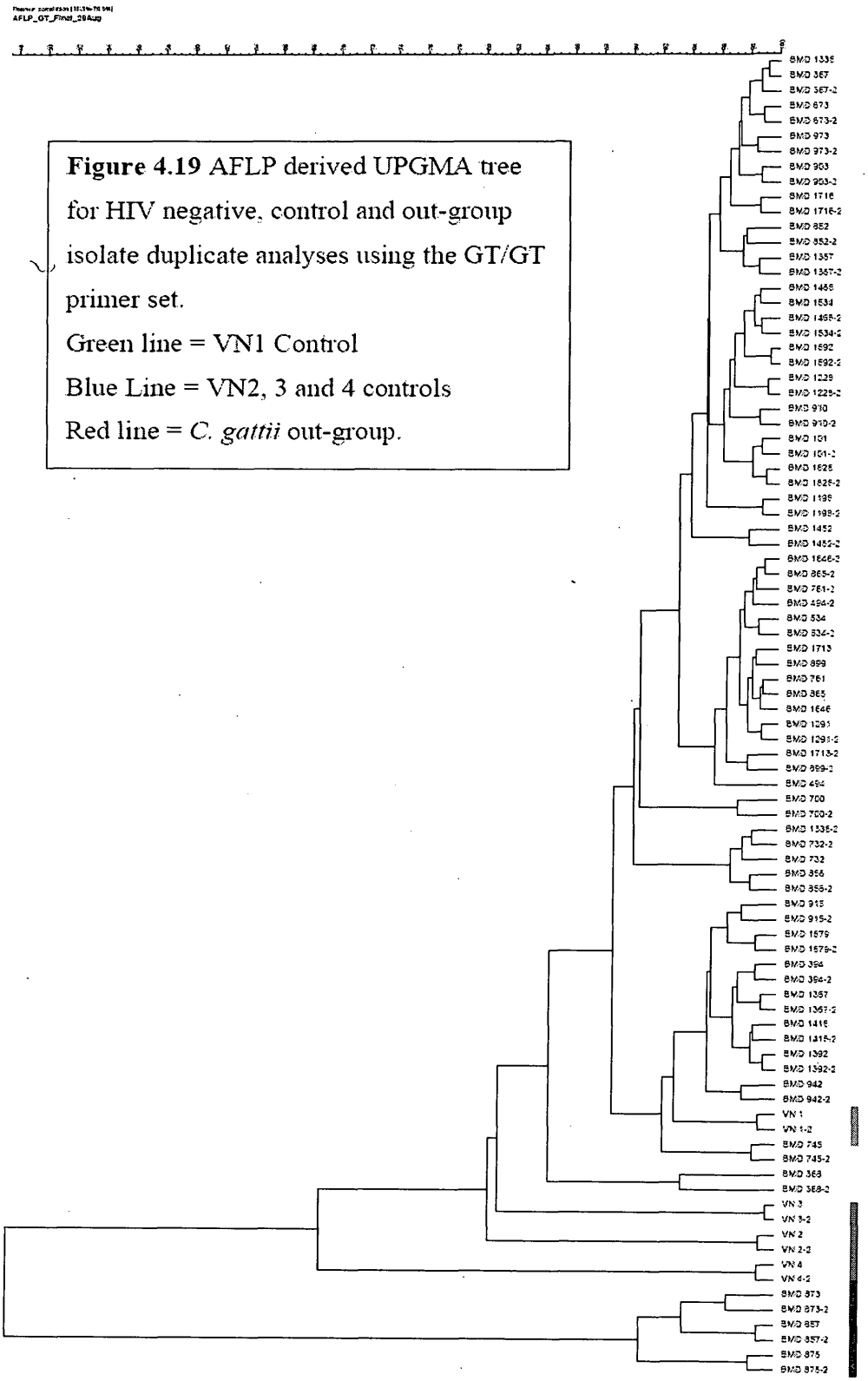


Figure 4.19 AFLP derived UPGMA tree for HIV negative, control and out-group isolate duplicate analyses using the GT/GT primer set.

Green line = VN1 Control

Blue Line = VN2, 3 and 4 controls

Red line = *C. gattii* out-group.

Table 4.5

Per cent similarity values between identical isolates for duplicate fragment analysis of HIV negative and control strains according to primer set used.

Primer set	Mean Similarity	95% Confidence Intervals	Standard Deviation	Median	10^{th} centile
AC/G	95.2	94.1 – 96.3	3.85	96.5	89.0
GT/GT	98.0	97.5 – 98.5	1.63	98.6	96.5

The variability introduced by fragment analysis was improved after gel and capillary replacement, now accounting for a median variability of 1.4 % between identical experiments for the GT/GT primer set, and 3.5% for the AC/G primer set. This artefactual variability introduced by the fragment analysis process is greater for the AC/G primer set compared with the GT/GT primer set. Given the information from the previous consistency experiment, AFLP is unlikely to be able to distinguish isolates with greater than 90 – 95% homology. This performance is similar to that reported in the literature [246, 307].

Having demonstrated reasonable performance of the typing system, I next analysed the whole dataset – controls, out-group, and strains from HIV infected and uninfected patients. The resulting trees are shown in figures 4.19 and 4.20.

Figure 4.20 NJT for 144 *C. neoformans* var *grubii* VN1 human isolates, AC/G primer set.

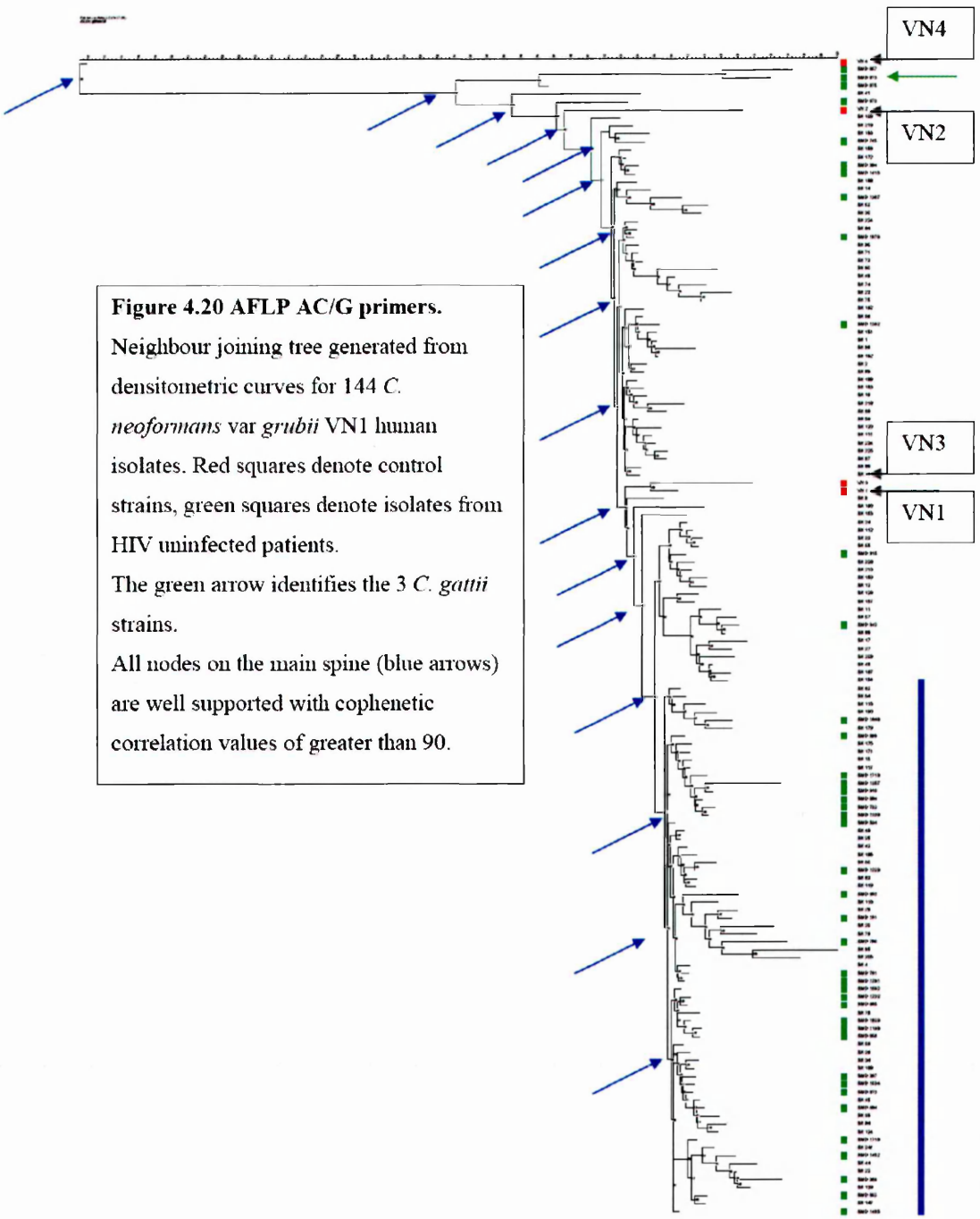


Figure 4.20 AFLP AC/G primers.
Neighbour joining tree generated from densitometric curves for 144 *C. neoformans* var *grubii* VN1 human isolates. Red squares denote control strains, green squares denote isolates from HIV uninfected patients.
The green arrow identifies the 3 *C. gattii* strains.
All nodes on the main spine (blue arrows) are well supported with cophenetic correlation values of greater than 90.

Figure 4.21 NJT for 144 *C. neoformans* var *grubii* VN1 human isolates, GT/GT primer set.

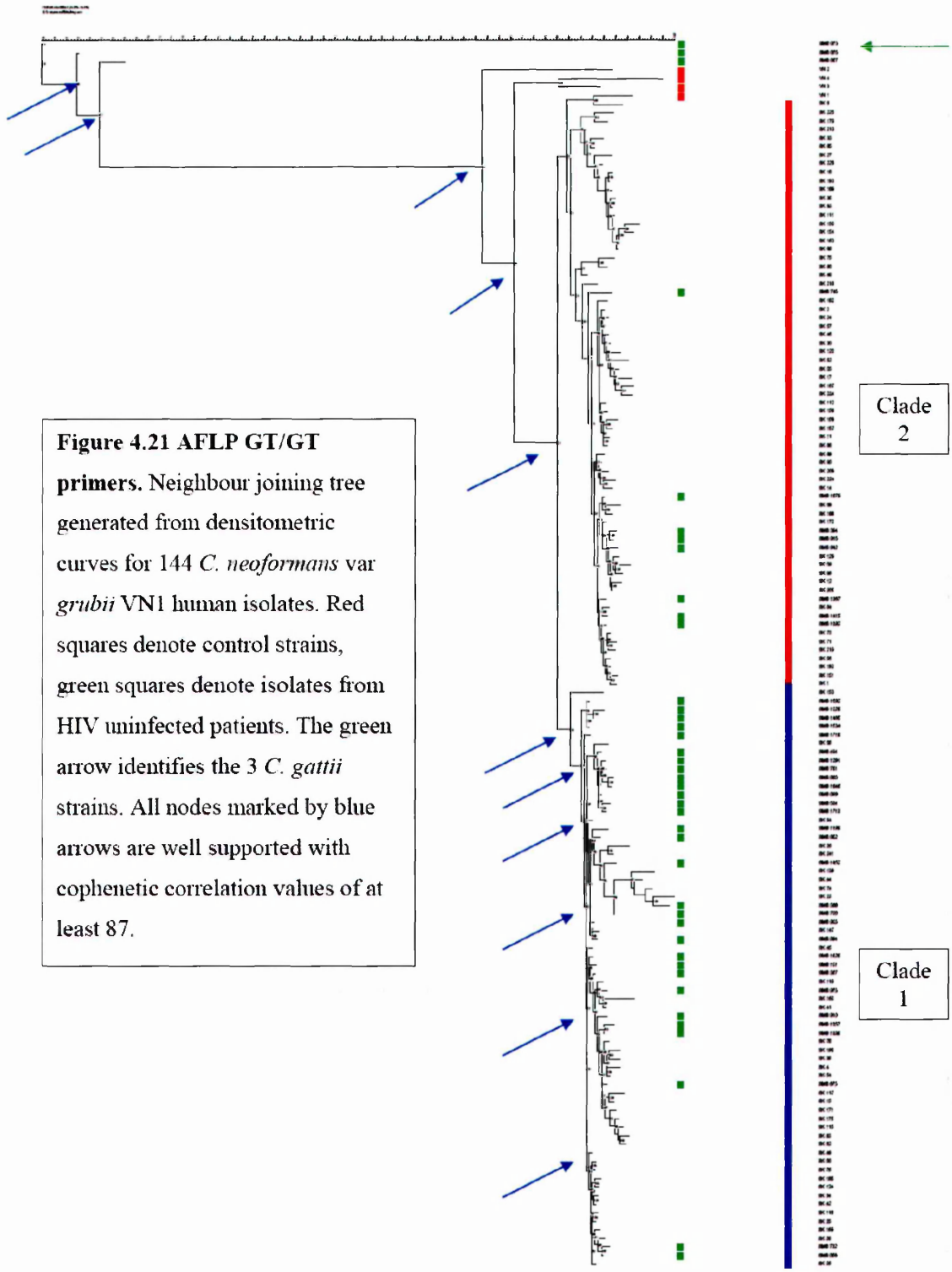


Figure 4.21 AFLP GT/GT primers. Neighbour joining tree generated from densitometric curves for 144 *C. neoformans* var *grubii* VN1 human isolates. Red squares denote control strains, green squares denote isolates from HIV uninfected patients. The green arrow identifies the 3 *C. gattii* strains. All nodes marked by blue arrows are well supported with cophenetic correlation values of at least 87.

Amplification with the AC/G primer set reliably separated the *C. gattii* out-group, and the Vietnamese isolates clustered with the VN1 control. All the major nodes along the tree were well supported with cophenetic correlation values of greater than 90. 30 of 39 isolates from HIV uninfected patients occurred in a monophyletic cluster marked by the blue line in the tree. The cophenetic correlation value of the node defining this cluster was 93.

Amplification with the GT/GT primer set also reliably defined the out-group and clustered the Vietnamese isolates with the VN1 control strain. Subsequently the population divided into two well supported main clades. The population of clade 1 consisted of 68 isolates, of which 30 were from HIV uninfected patients. Clade 2 consisted of 69 isolates of which 8 were from HIV uninfected patients. The distribution of isolates from HIV uninfected patients is shown in table 4.6 below.

Table 4.6
Distribution of isolates between clades by HIV status of source (GT primer set) (controls excluded).

Clade	HIV infected	HIV uninfected	Total
Clade 1	38	30	68
Clade 2	61	8	69
Total	99	38	137

78.9% of isolates from HIV uninfected patients were from clade 1 (odds ratio 5.93, 95% confidence interval 2.4 – 16.6, $p<0.0001$). The HIV uninfected strains in clade 1 were

the same strains as those in the monophyletic cluster identified by the AC/G primer set. Five strains from HIV infected patients identified in clade 2 by the GT primer set were not preserved in the monophyletic clade using the AC/G primer set, and 3 HIV associated strains that the AC/G primer set identified within the monophyletic clade were assigned to clade 1 by the GT primer set.

I used the automated band assignment algorithm in Bionumerics 5.1 to assign band classes for the experiments with each primer set. Dendrograms created using band classes can be tested with bootstrap analysis. The band assignment algorithm contains a tolerance setting – this defines the amount of gel distance that a band can have 'drifted' from a defined band class and still be classified as belonging to that class. This drift is described as a percentage of the total gel length. Since the automated sequencer can theoretically detect a difference in fragment length of a single base pair, and I knew the percentage of the gel which the size standard (consisting of 466 base pairs from 35 to 500) spanned, I was able to calculate that a single base pair change in length would account for approximately 0.14% of the gel length. I therefore set the tolerance value to be 0.07% (approximately +/- 0.5bp), which would enable the algorithm to assign bands to the nearest whole base pair length. The approach in assigning band classes used by other researchers is to define bands as having to be intense and polymorphic[246, 307]. The reason for using polymorphic bands is self-evident; however, how an intense band is defined is subjective, particularly if manual visual scoring is used. Bionumerics 5.1 overcame this problem to some extent by performing automated scoring once parameters have been defined, and thus removed the potential for bias in determining whether a band meets the intensity criteria or not (although bands can still be manually added or

removed by the operator after the automated step). However, the investigator still has to make a subjective decision regarding the range of intensity of bands that will be considered for scoring. Intensity is defined as a particular percentage of the most intense band on the gel. The level that defines intensity is chosen by the operator. I set the band defining algorithm to include all bands with at least 70% intensity of the maximum intensity for the GT primer set, and 20% for the AC primer set. The level was set lower for the AC primer set because there were few bands with 70% intensity. I then selected only the bands that were polymorphic. Using these parameters, 54 bands were generated for the AC/G primer set, and 63 bands for the GT primer set. I wanted to generate a large number of bands, because I suspected that my isolates were all closely related. The most diverse cryptococcal populations are African [307, 314]. Other investigators have found that using AFLP with 2 primer sets generating approximately 35 band classes each is sufficient to define genotypes in diverse populations [34, 315]. Since my strains were likely closely related, of a single molecular type and from a single geographic location, I thought that 35 bands might not be sufficient to distinguish the sub-populations present. I chose an intensity parameter that generated approximately twice the number of bands used by those investigating diverse populations. Choosing parameters that generated fewer fragments resulted in less ordered trees, but little extra was gained by increasing the number of bands.

Once band classes are created, the programme creates a matrix for each primer set where each isolate is scored for the presence or absence of the defined band classes. The Dice coefficient is used to generate neighbour joining trees from the binary matrices created. Figures 4.21 and 4.22 show the NJTs for the AC/G and the GT primer sets respectively.

Figure 4.22 NJT derived using the Dice coefficient with 54 bands defined from the AC/G primer set.

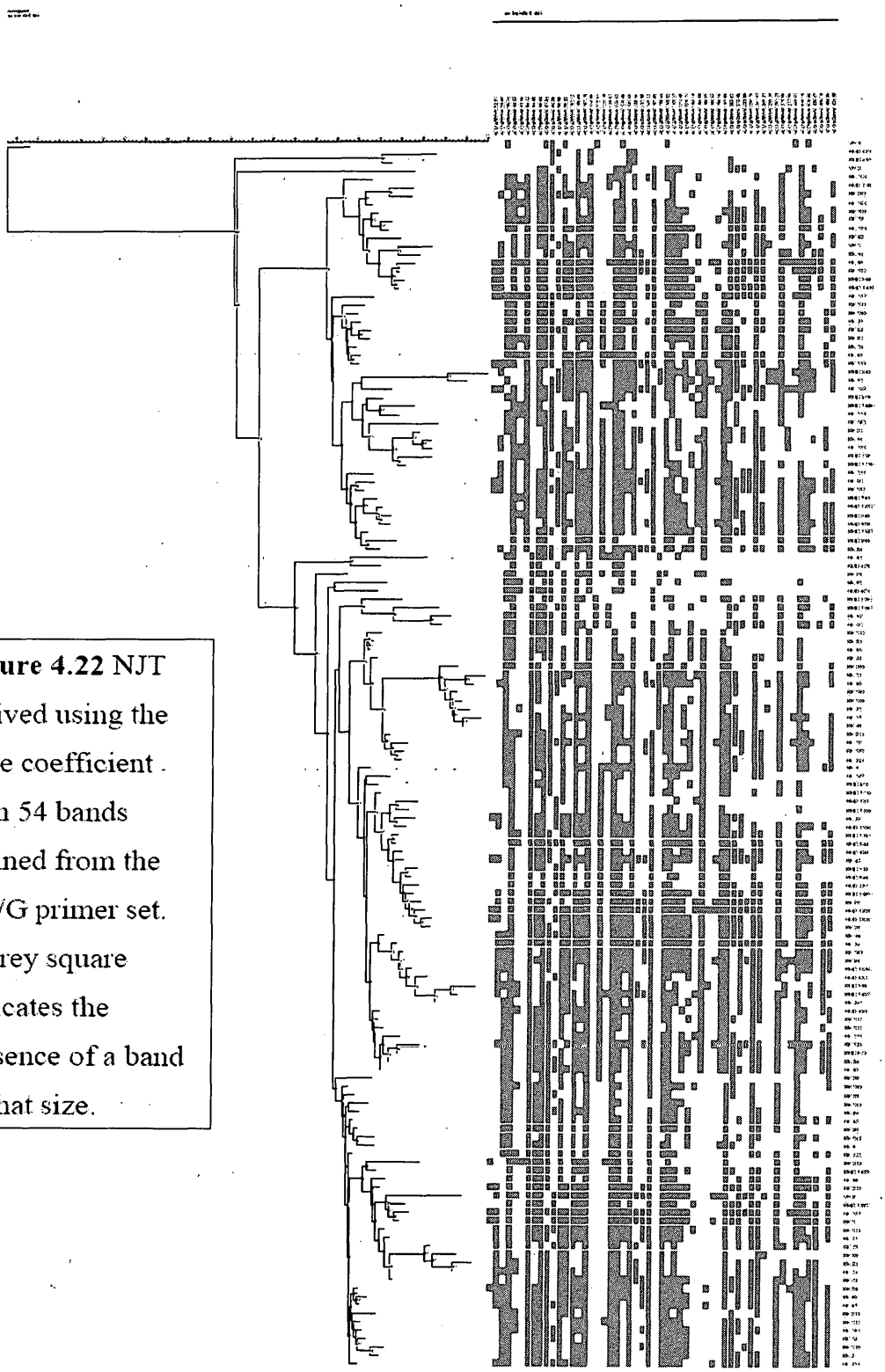


Figure 4.22 NJT derived using the Dice coefficient with 54 bands defined from the AC/G primer set. A grey square indicates the presence of a band of that size.

Figure 4.23 NJT derived using the Dice coefficient with 63 bands defined from the GT/GT primer set.

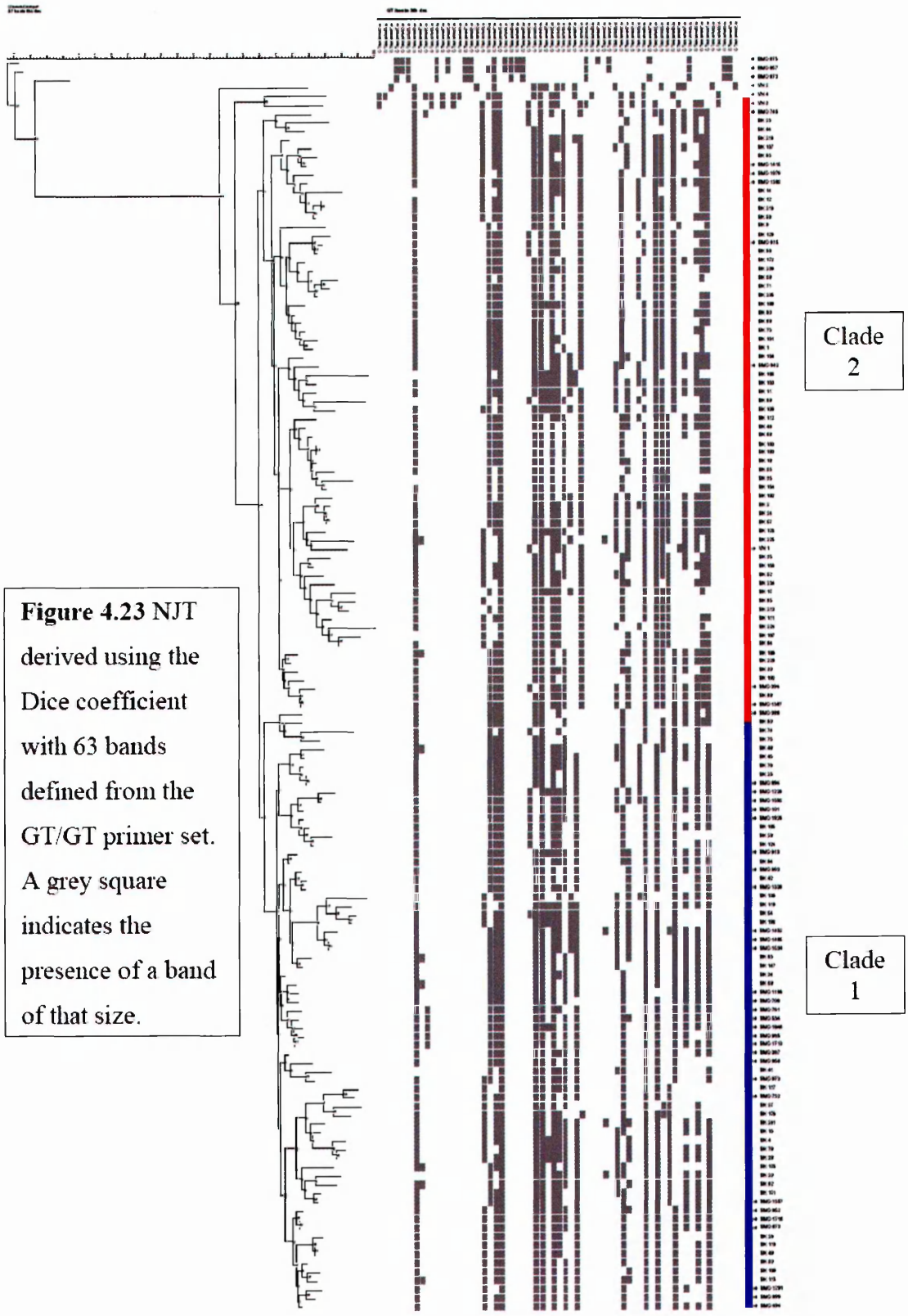


Figure 4.23 NJT derived using the Dice coefficient with 63 bands defined from the GT/GT primer set. A grey square indicates the presence of a band of that size.

Bootstrap analysis (100 iterations) was used to determine the statistical significance of the clusters generated. In the AC-derived tree, 2 main clusters were defined. The HIV negative isolates were more evenly spread between the two main clusters, accounting for 14 of 45 strains in the upper clade and 25 of 96 strains in the lower division (odds ratio 1.38, 95% confidence intervals 0.59 – 3.19, p=0.42, Fisher's exact test). Furthermore, the previous clustering of HIV negative strains was disrupted, with HIV negative strains transferring from cluster to cluster. Of particular note, one *C. gattii* strain (confirmed through *URA5* PCR-RFLP and biotyping) was now clustered with the *C. neoformans* var *grubii* strains, suggesting there had been significant information loss during the band matching process. As might be expected, the bootstrap values to support the divisions in the tree were weak, the node at the division of the VN1 clusters being generated in only 16% of iterations. No cluster was well supported other than for the node dividing the out-group (bootstrap value 100%).

With the GT band-based tree, 2 major clusters were created. The tree more closely resembles that generated by the comparison of the densitometric curves. The same HIV negative isolates are preserved within the same clusters (9 of 38 occurring in clade 2 compared with 29 of 38 in clade 1 (odds ratio 5.19, 95% CI 2.11 – 13.89, p<0.0001, Fisher's exact test). However, the bootstrap value at this node was low at 45%. All *C. gattii* strains were correctly sorted, although one might have expected the bootstrap value to be higher than the calculated 80%.

Band classes were identifiable that appeared to be highly discriminatory for either clade 1 or clade 2 HIV negative isolates, occurring almost exclusively in one or other clade. There were 4 such bands for each primer set. If used in isolation, as expected they

produced robust dendrograms. However, this approach is hardly scientifically valid, and illustrates the bias that can be introduced by manually assigning/scoring bands. If combined with other bands that had similar intensities in the whole population, the resulting dendrograms were no more robust than those presented in 4.19 and 4.20 (which indeed include the 8 discriminatory band classes).

4.6.4 Clinical Correlates

The HIV patients from whom strains were derived from this work are receiving different therapies as part of a randomised controlled trial which is not yet completed and thus cannot be analysed. Treatment, along with markers of disease severity at presentation, would need to be adjusted for in any analysis on the impact of clade. However, some of the isolates are from the patients described in Chapter 3. The infecting clade and fungal burden at baseline was known for 19 of the HIV infected patients in the dataset described in chapter 3. An exploratory analysis of the HIV infected patients reveals that there was no difference in the mean yeast colony forming count in the CSF of patients in clade 1 compared with those in clade 2 (5.8 log10 versus 6.1 log10, $p = 0.5$, 95% Confidence Interval for difference between the means -1.3 to 0.63, Welch 2 sample t-test). 11 of 19 HIV patients with clade 2 infections died compared with 4 of 10 with clade 1 infections ($p = 0.45$, Fisher's exact test).

Of the HIV negative patients, the clinical dataset is complete for only 22, of whom 19 were infected with clade 1 isolates. There was 1 death due to a clade 2 infection and 4 due to a clade 1 infection ($p = 1.0$, Fisher's exact test). Interestingly, only 4 of 19 patients with clade 1 infection had underlying disease compared with 3 of 3 patients

with clade 2 infection (p = 0.02, Fisher's exact test), supporting the hypothesis outlined in the introduction that there are some strains with increased virulence. The CSF cryptococcal antigen was higher for clade 2 strains compared with clade 1, but this difference was not statistically significant (clade 1 log10 CrAg titre = 2.2, clade 2 = 3.1, 95% CI for difference between means -3.96 to 2.14, p=0.37, n = 22).

4.7 Discussion

I have shown in Chapter 3 of the thesis that most HIV uninfected patients with cryptococcal meningitis in Viet Nam have no underlying immune deficit and that most disease is due to *C. neoformans* var *grubii*. The aim of this chapter was to determine the molecular epidemiological relationship between strains of *Cryptococcus neoformans* causing meningitis in HIV infected and uninfected patients, the underlying hypothesis being that the strains infecting HIV negative patients have relatively increased virulence, and would form a distinct population.

Until now there has been no detailed study of the molecular epidemiology of *Cryptococcus* spp from Viet Nam. I found that the vast majority of infections in our patients, irrespective of HIV serostatus, are caused by *C. neoformans* var *grubii URA5* PCR-RFLP group VN1. At the time of AFLP typing, RFLP typing had been completed on 53 isolates from HIV uninfected patients, and 211 isolates from HIV infected patients. 14 of these were *Cryptococcus gattii*. Subsequently I have completed RFLP typing of a total of 366 *Cryptococcus* spp, and have identified just 2 further *C. gattii* isolates, and one *C. neoformans* var *grubii* molecular group VN2. The HIV status of the sources of these 2 further *C. gattii* isolates is not known. I have not found any cases of

C. gattii in HIV positive patients. *C. gattii* thus accounts for less than 4% of cases of cryptococcal meningitis in our hospital.

I wanted to determine the relationship between strains infecting HIV uninfected patients in relation to strains infecting HIV infected patients using a typing method with high resolution. Having identified that the majority of strains in HIV uninfected patients were also *C. grubii* VN1, I used AFLP with 2 primer sets to investigate the relationship between these VN1 strains. Using the AC/G primer set, which would be expected to generate approximately 4 times as many fragments as the GT/GT primer set, I showed that most strains from HIV uninfected patients appeared to be more closely related to each other clustering in one part of the dendrogram. All nodes on the dendrogram were well supported with high cophenetic correlation values, showing that the tree closely represented the true similarities calculated between isolates. I used the neighbour joining tree method, which is considered to be consistent under many evolutionary models, and more reliable than other models such as UPGMA. Most HIV negative isolates formed a monophyletic clade, being derived from a common ancestor. As would be expected, this clade also included strains from HIV infected individuals (HIV infected patients would not be expected to be protected from infection with this clade).

The GT primer set, which appeared to be slightly more consistent than the AC/G primer set in repeated experiments on identical isolates, revealed 2 clades of *C. neoformans* var *grubii* VN1. Of the 137 Vietnamese strains, 30 of 38 strains from HIV uninfected patients (78.9%) were in clade 1 (odds ratio 5.93, 95% confidence interval 2.4 – 16.6, $p<0.0001$). A possible explanation for the different distribution in HIV negative strains

between clades is that strains in clade 1 are more virulent, and more able to cause disease in HIV uninfected patients.

Of note, in our dataset (99 HIV associated strains and 38 non-HIV associated strains) both clades 1 and 2 contain approximately the same total number of isolates – thus there are more HIV associated strains in clade 2 than in clade 1. If clade 1 strains really are more virulent, and the rate of exposure to each clade is equal, then one would expect clade 1 to also be the dominant strain in HIV infected patients The fact that it isn't (in fact, it is outnumbered approximately 2:1) suggests that exposure to clade 2 strains is more common than exposure to clade 1 strains. This is a pattern seen already with *C. gattii*. In Viet Nam, where the HIV seroprevalence rate in adults aged 15 – 60 is 0.5%, we have had had no definite cases of *C. gattii* infection in HIV infected patients even though we see disease in the immunocompetent, and a similar experience is reported from Africa, where the HIV prevalence is much higher [63, 70, 85, 87-89, 316, 317]. *C. neoformans* var *grubii* has been seen to replace *C. gattii* as the dominant human isolate as the HIV epidemic has spread [89]. For rare pathogens, where the ability to cause disease is not related to HIV status, one would expect to see more disease in the HIV uninfected patients where the HIV prevalence is low, since there is a greater chance of this group being exposed. Different exposures to the different clades is biologically plausible - *Cryptococcus gattii* occupies a different ecological niche to *C. neoformans/grubii*, and is well recognised to be much less prevalent in the environment [35, 48, 286, 318-321]. Since *Cryptococcus* species are not primary pathogens (unlike, for example, *Salmonella enterica* serovar Typhi or *Plasmodium falciparum*), differences in pathogenicity likely represent different adaptations to particular environmental niches,

rather than adaptations primarily to facilitate human disease. The prevalence and site of that niche in the environment will determine the risk of a potentially infectious contact between the pathogen and susceptible hosts (for example, as might be expected, Koala bears are prone to infection with *C. gattii*). Our data suggests that amongst HIV infected patients exposure to clade 2 strains is at least twice as common as exposure to clade 1 strains, and could be higher still if clade 2 is also less able to cause disease in HIV infected patients.

Alternatively, the higher number of clade 2 infections in HIV patients might be the consequence of a particular exposure that is more common in HIV patients. For example, a large number (62.8%) of our patients with HIV associated cryptococcal meningitis are intravenous drug users. However, I found no difference in intravenous drug use rates amongst HIV positive patients by clade (21 of 37 versus 33 of 52, $p = 0.84$). The mode of acquisition of cryptococcal disease is not known, although it is thought inhalation of desiccated yeast cells or basidiospores is the most likely explanation [4]. Whether disease represents primary infection or is the result of recrudescence of latent infection is also a matter of debate (both are described), although clearly the incubation period can be prolonged [73, 74].

A further possible explanation, and one that has been postulated for *C. gattii* in the past, is that Clade 1 organisms exploit some other 'gap' in the immune system that is not currently recognised. [322].

It could be that the clades created are artefactual, and a consequence of error somewhere in the experimental process. AFLP is a multi-stage procedure, and while reportedly reproducible, error could enter at any stage, for example through different efficiencies of

digestion, ligation, PCR amplification or fragment detection [280, 281, 323]. I performed multiple experiments on single strains, as described, to determine which methods of digestion and ligation were the most consistent. In order to further reduce the effect of experimental error, I randomised the selection of strains for experimentation. The strains were randomly assigned to batches of 24, and experiments were done by batch from DNA extraction through to the final round of selective amplification. Because during the optimisation process I identified that the sequencer fragment analysis was a potential source of experimental error, with variation in fragment detection by sequencer run, all samples were run at the same time, with fresh gel and capillaries, and the controls and HIV negative strain experiments were run in duplicate (1 copy on each 96 well plate), to enable us to detect any significant sequencer variability by plate in the final experiment. All fragment files were updated at the same time to Bionumerics 5.1 for normalisation. I am confident I have taken as many steps as possible to ensure experimental consistency. While the tree structure is slightly different for each primer set, the location of strains within the trees is similar, suggesting that the clustering of HIV negative isolates that I have found is genuine. Furthermore, the description of a homogeneous population of *C. neoformans* var *grubii* VN1 recently reported from China supports the validity of my findings [273].

The low values in the bootstrap analysis are in contrast to the high cophenetic correlation values seen at the nodes in the trees generated from the densitometric curve analyses. The cophenetic correlation values measure how accurately the derived similarity values of the dendrogram reflect the true similarity values. The bootstrapping process is an attempt to measure the influence different band classes have on the shape .

of the dendrogram. This is achieved by randomly sampling band classes from the defined band set. Sampling is with replacement – i.e. a single band class can be repeatedly added to the bootstrap experiment. Bands are sampled until the number of bands sampled in the iteration equals the number of bands defined in the dataset. The dendrogram is repeatedly constructed using the Dice coefficient [309]. At least 100 iterations are performed, and the number of times that a particular node is reconstructed is expressed as a percentage of the total number of iterations. The low bootstrap values in my dataset were disappointing, but may reflect the fact that the isolates are closely related, and that differences within clusters are due to numerous polymorphic bands that occur with low frequency in the dataset. Within a cluster, each strain may differ by only 4 or 5 bands, but the bands that differ are unique for each strain (i.e. are present rarely in more than one strain). In other words, while the total number of band class differences between strains in a cluster is low, these differences can be spread over a large number of band classes. For example, in a comparison of strains that had >92% similarity to a randomly chosen "reference" strain, I found 107 band classes of which 47 were polymorphic - meaning that the average difference of about 5 bands between any given combination of two patterns was spread over these 47 classes. Thus each bootstrap iteration is unlikely to result in the same cluster pattern for closely related strains. However, very different strains, such as the out-group, which were *C. gattii*, are robustly clustered. Where the strains are closely related, the band class defining process is associated, as might be expected, with a significant loss of information. It is akin to artificially dichotomizing continuous data, and furthermore is subject to the bias of the investigator, particularly where bands are scored manually. However, the densitometric

curve comparison process is free of operator interference. Information is also potentially lost in the band matching analysis since the presence/absence of bands is digitally scored, when in fact it is plausible that the difference in intensity of different bands has scientific meaning, because of homoplasy. The densitometric curve analysis takes into account differences in intensities between peaks within a profile and between isolates. While AFLP has the advantage of being a whole genome based typing system, the information drawn from fragment patterns is limited because the exact cause of different banding patterns is not known. Bands of different sizes may contain more than one sequence (homoplasy) that the technique cannot distinguish. It is not possible to infer exactly how an alteration in a cut site will alter the banding pattern. It is not possible to draw the same inferences about evolution that can be drawn with sequence data, and it is more difficult to confirm consistency.

Host and geographical factors have been shown to influence the species of *Cryptococcus* which cause meningitis in particular groups. However, a sub-group of *C. neoformans* var *grubii* that is associated with HIV uninfected individuals, such as I have shown, has been described only once before, despite extensive study [64, 80, 287, 324]. Boekhout used AFLP to genotype 207 isolates from all continents (except Antarctica) including human, animal and environmental isolates, and described 3 AFLP genotypes within the *C. gattii* and *C. neoformans* (including *C. neoformans* var *grubii*) species [34]. All isolates had unique AFLP banding patterns. He found no association by AFLP genotype with HIV serostatus, but the genotypes were defined at the varietal level (var *neoformans*, var *grubii* and a hybrid clade). Of note he found no geographical substructure to *C. neoformans* var *grubii* strains, but he had only 4 strains from Thailand

in his collection, and none from elsewhere in east and Southeast Asia. There were no differences in the genotypes of strains from HIV infected and uninfected patients. Litvintseva used AFLP to characterise environmental and clinical isolates of *C. neoformans* in North Carolina, and described 2 genotypes of *C. neoformans* var *grubii* [315]. No differences in genotype distribution were found by HIV serostatus of the isolate source. Meyer used minisatellite typing to determine the relationship of Ibero-American isolates of *Cryptococcus* [35]. Of 266 clinical isolates, 139 were *C. neoformans* var *grubii* VN1 from HIV infected patients, and 21 were uninfected patients. No particular *C. neoformans* var *grubii* genotype was described as being associated with HIV uninfected strains. However, in 1995 Varma had described using a genomic probe UT-4p to create DNA fingerprints of *Cryptococcus neoformans* including 25 isolates from patients with HIV and 23 from patients without HIV [324]. Nine fingerprints were described for serotype A strains, and isolates from HIV uninfected patients were more likely to be genotype 2 than other genotypes. No particular association with genotype was found for isolates from HIV patients. More recently, it has been reported that in 91 apparently immunocompetent patients with cryptococcal meningitis in China, disease is due to infection with a highly homogeneous group of *C. neoformans* var *grubii* VN1. The strains were collected from 16 provinces over a 26 year period until 2006. All strains were found to be of a single (8 locus) MLST genotype. However, no comparison of genotype was made with strains from HIV infected patients. In a mouse infection model, 4 of these Chinese strains were found to have variable virulence – one was similarly virulent to the hypervirulent H-99 strain, one had similar virulence to the VN1 reference strain, and 2 had intermediate virulence,

suggesting that there may be wide variation in the virulence of this particular strain [273, 325]. Alternatively, there may be subpopulations of increased virulence that the MLST scheme used could not resolve. However, the validity of the mouse model in relation to human disease has not been determined [273].

In our patients, other than having an increased ability to infect HIV uninfected patients, it is currently difficult to determine whether clade 1 strains cause more severe disease because clinical data is not complete or is embargoed. The HIV patients from whom strains were derived from this work are receiving different therapies as part of a randomised controlled trial which is not yet completed and thus cannot be analysed. Treatment, along with markers of disease severity at presentation, would need to be adjusted for in any analysis on the impact of clade. However, an exploratory analysis using the clinical data presented in chapter 3 suggests that fungal load in HIV patients is independent of infecting clade, and that there is no difference in the 10 week outcome. For HIV uninfected patients, underlying disease was more common in patients with clade 1, supporting the hypothesis that this is better able to cause disease in the immunocompetent, although differences in fungal burden, estimated by cryptococcal antigen titre, were not statistically significantly different.

4.8 Conclusion

Classical teaching would have suggested that, in our tropical location, more cases in HIV uninfected patients would have been due to infection with *Cryptococcus gattii* [76, 269]. *C. neoformans* var *grubii* infection, the commonest cause of cryptococcal meningitis worldwide since the HIV epidemic, most frequently occurs in patients with

an underlying immunosuppressive condition[61, 62, 270, 271]. I may have identified a clade of *C. neoformans* var *grubii* that has increased ability to infect immunocompetent patients. This clade is not the predominant cause of cryptococcal meningitis in HIV infected patients in Viet Nam, suggesting exposure is less frequent than to clade 2. At the time of writing, the available clinical dataset is not sufficient to describe differences in clinical phenotype to clade, but I will be able to answer this question in the future. Additional evidence of increased virulence would need to be obtained from animal studies. However, the result of AFLP typing is not conclusive – the use of a sequence based typing method, such as MLST, may confirm the result, would allow more robust statistical analysis and would allow comparison with genotypes from other geographical areas. Next generation whole genome sequencing of multiple strains would provide the definitive answer. Current DNA based genotyping methods for typing *Cryptococcus* species offer a limited and varying degree of subspecies, clade and strain level discriminatory power. Whole genome sequencing is the most accurate and reliable method to identify, type and determine phylogenetic relationships among strains of a species. Additionally, whole genome sequence based SNP information gained from a representative population of strains may be used to perform evolutionary comparisons of strains, or selection of unique strains for whole genome sequencing projects. As the cost of sequencing falls with newer technologies, the approach is becoming feasible, as has been demonstrated already for some pathogens [326-328]. The same approach should be developed for *Cryptococcus* species.

Chapter 5

Antifungal susceptibility testing and *Cryptococcus neoformans.*

The aims of this chapter of the thesis are as follows:

1. To describe the antifungal susceptibilities of Vietnamese *Cryptococcus* clinical isolates to 7 antifungal agents, and to determine whether the susceptibilities have changed over time, vary by host HIV serostatus or by species.
2. To describe the correlation between susceptibility to different azoles.
3. To determine whether differences in amphotericin B susceptibility correlate with the rate of fungal clearance from cerebrospinal fluid.
4. To determine whether the results of antifungal sensitivity testing are associated with clinical outcome in patients with cryptococcal meningitis.

5.1 Introduction

The ideal testing system for determining the susceptibility of a microbe to a given antimicrobial drug would provide information that predicts the clinical outcome (or at least the sterilising or antimicrobial effect in the patient) when that particular drug is used. In addition, the test would be cheap, reliable, have high inter-user and inter-laboratory consistency, and be simple to perform and interpret, generating results quickly enough to allow meaningful management interventions. The increasing prevalence of invasive fungal infections together with the discovery of new antifungal agents has seen the development of numerous methods for determining the antifungal

susceptibility of fungal pathogens [113, 159, 160, 162-164, 166, 167, 329-346]. The gold standard for yeasts, against which most other testing systems are measured, is the Clinical and Laboratory Standards Institute (CLSI, Wayne, PA) protocol M27A-A2 [169]. This protocol is a liquid medium-based microbroth methodology using 96 well plates. Plates are not commercially available, but must be made up in house using pure drug, and prepared plates need to be stored at -80*C*. Growth in each drug dilution is estimated by using a spectrometer to measure the turbidity in each well. The MIC of drug for a particular isolate is that at which the turbidity is 20% of the positive control. The disadvantages of the method include the following: plate preparation is time consuming, there can be inter-laboratory variation in the quality of plates produced, plates have to be stored at -80°C and measurement of the MIC requires a spectrophotometer. However, the major limitation of the methodology is that it has not been demonstrated that it reliably predicts clinical outcome in cryptococcosis [178]. The Sensititre® YeastOne® (Trek Diagnostics, East Grinstead, UK) susceptibility testing system is a commercially available microbroth method that enables measurement of MICs of 8 licensed antifungal drugs. It uses a 96 well plate format, and an indicator (alamar blue) demonstrates the presence or absence of growth, enabling assessment of the MIC by the naked eye and without the need of a spectrophotometer. Centralised manufacturing ensures plate consistency, plates are robust and can be stored at room temperature, and reading the result is simple with the naked eye. This ensures consistency of measurement between laboratories [347]. MICs measured for azole drugs have good correlation with the results obtained by the CLSI M27A methodology, but correlation with amphotericin B MICs, particularly for *Cryptococcus*, is poorer[167].

Given that amphotericin B MICs determined by the CLSI method have not been shown to be clinically relevant, this may not be a disadvantage. The failure to show a relationship between MICs and outcome in cryptococcal meningitis may be because the *in vitro* methodologies are poor models of the *in vivo* reality, or may be a reflection of the quality of the studies investigating this relationship. Most studies investigating this relationship have been small (25 – 96 patients), from case series or do not account for differences in disease severity at baseline [161, 182, 183, 348, 349]. The ideal data to investigate the relationship between MIC and outcome would come from randomized controlled trials, since it removes bias in treatment allocation. Additionally, baseline indicators of severity, such as those identified in Chapter 3, would need to be accounted for in the analysis. However, even in the absence of data correlating with clinical outcome, in vitro susceptibility testing may have a role in surveillance and in detecting differences in susceptibility between strains and species that may provide insight into mechanisms of drug action and the development of resistance. In this chapter of the thesis I use Sensititre® YeastOne® antifungal susceptibility test plates to describe the susceptibilities of Vietnamese clinical isolates of *Cryptococcus*, and relate this to HIV, time, species, clade, rate of clearance of yeast from the CSF and clinical outcome.

5.2 Methods

5.2.1 Determination of Minimum Inhibitory Concentrations

I used the Sensititre® YeastOne® (Trek Diagnostic Systems, East Grinstead, UK) microbroth susceptibility testing plates, as described in Chapter 2, to determine the

MICs of 8 antifungal drugs against all the *Cryptococcus* species in the OUCRU strain collection.

5.2.2 Isolates

The isolates tested were derived from 3 sources: the BMD study (a prospective descriptive study of CNS infection in HIV uninfected patients); the BK study (a prospective randomized controlled trial of combination antifungal therapies in cryptococcal meningitis in HIV infected patients); and a collection of arcHIVed ('A-strain') cryptococcal isolates dating from 1996 until the start of the BK study in 2004. The BMD isolates dated from 1996 to 2009 (n = 60), the BK isolates from 2004 to 2009 (n = 214), and the A-strain isolates were collected between 1996 and 2003 (n = 128). There were no clinical data for this last group of isolates, but they are almost certainly all isolates from HIV infected patients - since 1996 all HIV uninfected patients with cryptococcal meningitis at the Hospital for Tropical Diseases were cared for on the CNS infections ward (formerly the Malaria Research Ward), and were recruited to the BMD study. Patient names were available for the A-strain patients, and cross referencing with the BMD database revealed that there was no strain duplication.

5.3 Analysis

5.3.1 Antifungal Susceptibility

The isolates were assigned to groups by source in relation to HIV serostatus - HIV uninfected (the BMD study strains), HIV infected (the BK study strains) and unknown HIV status (the A-strain). A group of strains where HIV infection was highly likely was

defined by combining all the strains from the BK study and the A-strain group. Strains were also assigned to groups according to the year of isolation.

All MICs were $\log_2$ transformed prior to analysis. Antifungal susceptibilities were described in 3 ways – as the Minimum Inhibitory Concentration 50 (MIC_{50}) – the concentration of drug which inhibited growth of 50% of the isolates; as the Minimum Inhibitory Concentration 90 (MIC_{90}) – the concentration of drug which inhibited growth of 90% of isolates; and as the geometric mean of the minimum inhibitory concentrations. The MICs were compared by year and groups using a linear model. If the plot of MIC by year suggested that the relationship was unlikely to be linear then the relationship was instead modelled with a natural cubic spline with 5 degrees of freedom and a global F test was used to assess whether the year affected the MIC. Natural cubic splines allow flexible modelling and test for very general (linear or nonlinear) associations [279]. However, as spline functions are relatively complex objects, the estimated association cannot easily be quantified by simple effect measures.

5.3.2 Correlation Between Azole MICs

Pearson's rank correlation was used to describe the relationship between the MICs of different azoles and fluconazole. Linear regression was used to determine the proportion of occasions on which the fluconazole 72 hour MIC exactly or closely (+/-1 dilution) predicted the MIC of the other azole drugs tested. Fluconazole was chosen as the reference azole, since it is the recommended and most frequently prescribed treatment for cryptococcal meningitis.

5.3.3 Relationship between Amphotericin B MIC and rate of clearance of yeast.

Quantitative counts were determined as described in Chapter 2. We used a nonlinear mixed effects model to model the decline of quantitative counts over time in the HIV patients described in chapter 3, who all received 4 weeks of treatment with amphotericin B 1mg/kg/day. We assumed that for each patient, the quantitative counts decline exponentially after treatment initiation, i.e. that the count for patient i on study day t is given by the relation $Count_i(t) \approx \exp(a_i + b_i \cdot t)$ where the coefficient $\exp(a_i)$ refers to the 'true' quantitative count (i.e. the quantitative count without measurement error) at therapy initiation (day 0) and $\exp(b_i)$ refers to the relative count decline per day of treatment. The quantitative counts (after addition of 1 to cope with counts of zero) were log-transformed to normalize their distribution. Thus, the non-linear patient-specific model assumed that $\log_e(Count_i(t)+1) \approx \log_e(\exp(a_i + b_i \cdot t)+1)$. In addition to this basic model we fitted an extended model where the patient-specific slope was allowed to change from day 14 onwards, i.e. we modelled the decline (on the logarithmic scale) with a linear spline function with one knot at 14 days.

The patient-specific intercepts and slopes (a_i, b_i) were modelled according to a bivariate normal distribution with average (population) intercept a and slope b plus normally distributed patient-specific deviations from these average values to describe between-patient variation. In the extended model, we additionally used a fixed (population) effect for the change in slope after day 14.

In order to assess whether the initial count or the rate of decline depended on the MIC's of amphotericin B or fluconazole (measured at 48 or 72 hours), respectively, we tested

whether the population slope a and intercept b (plus the change in slope after day 14 for the extended model) depended linearly on the log2-transformed MIC-values.

Finally, we tested whether the initial count or the rate of decline predicted the patients' 6-month survival. This was done with a joint model where the counts were modelled as described above and the survival endpoint was modelled with a Weibull proportional hazards regression model where the hazard was allowed to depend on both the patient-specific intercept and slope a_i and b_i [350]. A joint model where the counts followed the extended model could not be fitted due to numerical problems, i.e. the algorithm did not converge. We used a Weibull model instead of the more commonly used Cox proportional hazards model as the two often give comparable results and a joint model was easier to implement with a parametric survival model.

5.3.4 Relationship to clinical outcome

To determine the relationship between drug susceptibility and clinical outcome, I used the clinical dataset and isolates from the patients described in chapter 3. The Cox proportional hazards regression model, described in chapter 3, was used to study the association between MIC and survival. Initially a univariate logistic regression was performed to investigate the relationship between MIC and survival. I then performed a multivariate analysis including in the model the MIC of the antifungal drug of interest and the 3 variables identified in chapter 3 as being independently associated with an altered hazard for death (log2 CSF cryptococcal antigen titre, Glasgow Coma Score and CSF white cell count), since these might otherwise have obscured an association with outcome. All patients were initially treated with at least 2 weeks of amphotericin B

(1mg/kg/day) followed by fluconazole 400mg/day to complete 10 weeks treatment. Long term suppression was continued with fluconazole 200mg/day.

5.3.5 Statistical software

Statistical analyses were performed using R version 2.9.0 (R Foundation for Statistical Computing, Vienna, Austria)[247]. The rate of clearance of yeast was modelled using the recommended R package nlme [247, 351]. The joint model of yeast clearance and survival was programmed with proc nlmixed of the statistical software SAS 9.2 (SAS Institute Inc., Cary, North Carolina, USA).

5.4 Results

5.4.1 Antifungal Susceptibilities

402 clinical isolates were available for antifungal testing during period 1996 – 2009. The isolates are described in Table 5.2.

Table 5.1

Summary of number of samples tested by year and source HIV status

Year	HIV Uninfected (BMD Strains)	HIV Infected (BK Strains)	HIV status Unknown (A-Strains)	Row Total
1996	0	0	7	7
1997	1	0	8	9
1999	1	0	2	3
2000	4	0	22	26
2001	3	0	39	42
2002	3	0	50	53
2003	4	0	0	4
2004	1	34	0	35
2005	9	63	0	72
2006	8	52	0	60
2007	12	35	0	47
2008	8	26	0	34
2009	6	4	0	10
Column Total	**60**	**214**	**128**	**402**

The spread of MICs measured are described by the histograms in Figure 5.1 below.

Figure 5.1 Histograms depicting the distributions of MICs measured for the 8 antifungal drugs after 48 and 72 hours of incubation.

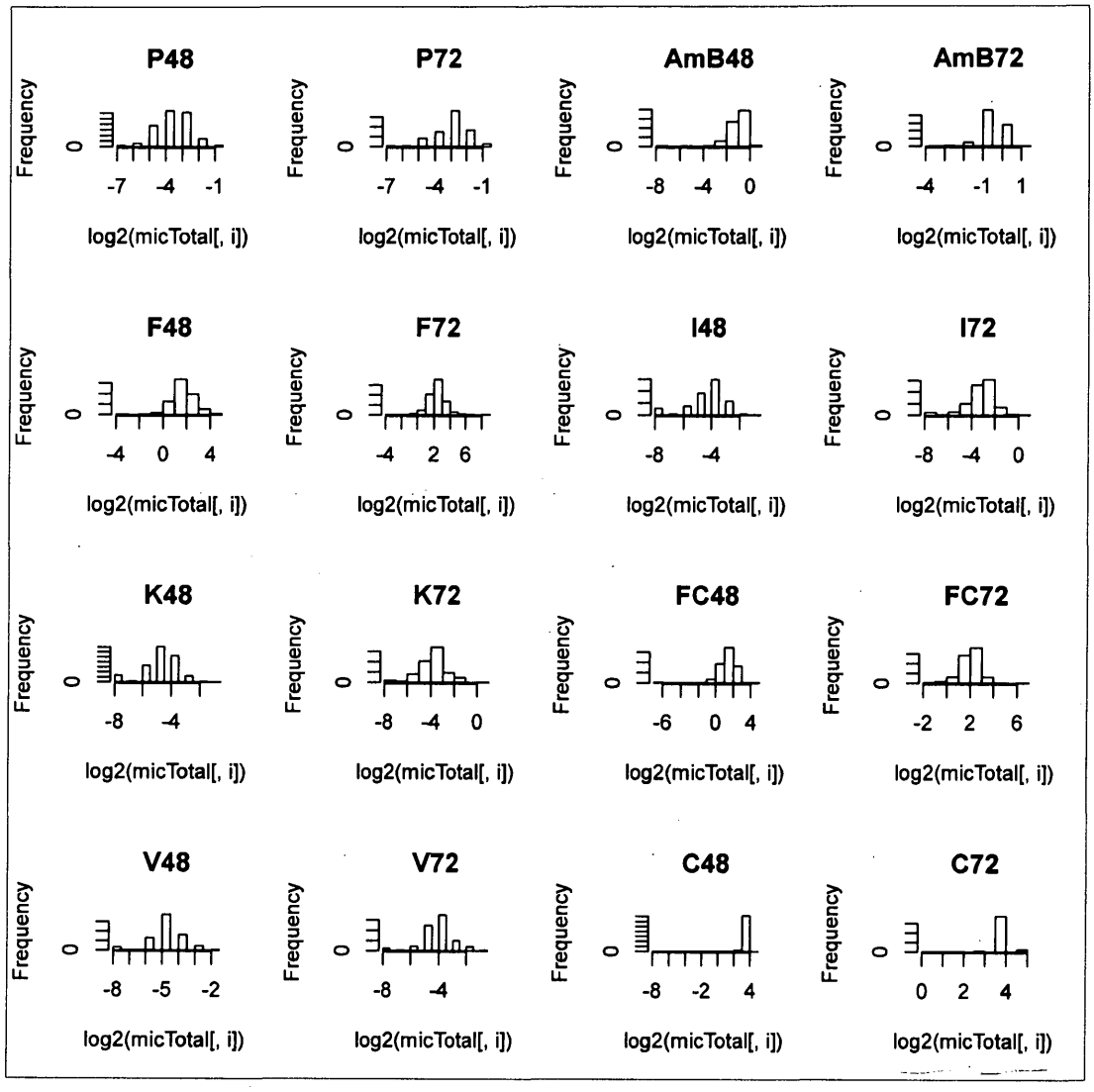


Figure 5.1 Legend: P = Posaconazole, AmB = Amphotericin B, F = Fluconazole, I = Itraconazole, K = Ketoconazole, FC = Flucytosine, V = Voriconazole, C = Caspofungin.
48 = 48 hours, 72 = 72 hours

The distributions of the $\log_2$ antifungal MICs are approximately symmetric with the exceptions of amphotericin B and caspofungin. The percentages of isolates susceptible

to each individual drug concentration at 48 and 72 hours are shown in Tables 5.3 and 5.4 below. Fewer results are available at 48 hours because of the slow growing nature of *Cryptococcus spp.* resulting in insufficient growth from a number of isolates to enable MIC estimation (despite an adequate inoculation density). As a point of reference, the CLSI M27A suggested interpretative breakpoints for *Candida* species are included in the table (shaded regions: green - sensitive; yellow - intermediate sensitivity; orange - sensitive dose dependent; red - resistant). Breakpoints for *Cryptococcus* are not established, but some investigators have used these in their analyses of *Cryptococcus* sensitivities[352].

The range of MICs detected for amphotericin B was narrow, with 92.6% of strains susceptible to concentrations of 0.256 or 0.512 ug/ml after 72 hours incubation. As expected for *Cryptococcus*, all strains tested were resistant to caspofungin (data shown in histograms only). Growth occurred across the whole range of flucytosine and fluconazole drug concentrations suggesting the presence of drug resistance. The strains for which MICs of fluconazole or flucytosine were in the defined resistance range for *Candida* species were all isolates from HIV positive patients from 2005 onwards (5 with fluconazole MICs>64mg/L, 2 with flucytosine MICs>32mg/L). The MICs of itraconazole for 2 isolates were 1 and 2 mg/L respectively (concentrations considered to denote resistance for *Candida*). One of these was a definitely HIV associated isolate from 2005, the other was a probably HIV associated isolate from 2000. Some strains from all groups of isolates had MICs that would be considered to represent intermediate resistance or a dose-dependent clinical response in *Candida*. The distribution did not appear to be different between groups.

Table 5.2 Percentage of strains with particular 48 hour MICs by antifungal agent

Drug	N	MIC ug/mL												
Amphotericin	371	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
B	%	0.5	0.3	0.3	0.8	7.5	36.4	52.6	1.6	0	0	0	0	0
Flucytosine	371	**0.03**	**0.06**	**0.125**	**0.256**	**0.512**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
	%	0	0	0.3	0	0.3	4.9	24.8	46.9	22.4	0.3	0.3	0	0
Fluconazole	372	**0.125**	**0.256**	**0.512**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**>256**
	%	0.27	0.27	0.27	2.4	17.2	45.4	26.1	7	1.1	0.75	0.25	0	0.25
Itraconazole	370	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
	%	8.4	10.0	24.6	40.0	15.7	1.1	0.3	0	0	0	0	0	0
Ketoconazole	372	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
	%	7.8	17.7	38.2	28.5	6.7	0.8	0.3	0	0	0	0	0	0
Posaconazole	372	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**>8**	**-**
	%	0.8	3.0	20.2	34.4	32.8	7.5	1.3	0	0	0	0	0	-
Voriconazole	370	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
	%	3.8	17.0	50.8	21.6	5.9	0.8	0	0	0	0	0	0	0

Table 5.3 Percentage of strains with particular 72 hour MICs by antifungal agent

Drug	N	MIC ug/mL												
Amphotericin B	402	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
	%	0	0	0	0.25	1.0	6.0	58.0	34.6	0.25	0	0	0	0
Flucytosine	399	**0.03**	**0.06**	**0.125**	**0.256**	**0.512**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**>64**
	%	0	0	0	0	0.25	2.25	8	35.6	45.4	8	0.25	0	0.25
Fluconazole	400	**0.125**	**0.256**	**0.512**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**>256**
	%	0.5	0	0	1.25	5.75	26.0	44.75	17.25	3.25	0.75	0.25	0	0.25
Itraconazole	399	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
	%	3.25	3.25	12.5	32.6	38.6	8.5	0.75	0.25	0.25	0	0	0	0
Ketoconazole	400	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
	%	3.25	10.25	25.5	43.0	11.75	6	0	0.25	0	0	0	0	0
Posaconazole	400	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**>8**	-
	%	0.75	0.75	10.5	18.5	45.5	21.0	3.0	0	0	0	0	0	-
Voriconazole	399	**0.008**	**0.016**	**0.032**	**0.064**	**0.128**	**0.256**	**0.512**	**1.024**	**2**	**4**	**8**	**16**	**>16**
	%	2.75	5.25	30.75	43.9	12.0	4.8	0.5	0	0	0	0	0	0

5.4.2 MIC_{50}, MIC_{90} and Geometric Mean MICs

The numbers tested, MIC_{50}s, MIC_{90}s and geometric means after 72 hours incubation are shown in table 5.4, according to HIV serostatus of the isolate source.

Multiple linear regression was used to test the effect of HIV serostatus on geometric mean MIC for each antifungal drug. Results for isolates from HIV infected patients were compared with 2 groups - HIV uninfected and patients with unknown HIV status. The coefficients in table 5.5 below give the estimated differences in geometric means of each group compared to HIV infected patients:

Table 5.4 Antifungal MICs by HIV status of source (ug/mL)

Antifungal		Patient Status: *HIV infected (BK) (214)*	*HIV uninfected (BMD) (60)*	*Unknown Status (128)*
Amphotericin B	MIC 50	0.512	0.512	0.512
	MIC 90	1.024	1.024	1.024
	Range	0.064 – 2.048	0.256 – 1.024	0.128 – 1.024
	Geometric mean	**0.690**	**0.60**	**0.512**
	95% CI Geometric mean	**0.650 – 0.732**	**0.544 – 0.666**	**0.476 – 0.551**
	N	214	60	128
Flucytosine	MIC 50	8	8	4
	MIC 90	16	8	8
	Range	0.48 - 128	0.96 - 16	0.96 – 16
	Geometric mean	**6.99**	**4.91**	**4.26**
	95% CI Geometric mean	**6.48 – 7.54**	**4.08 – 5.92**	**3.87 – 4.69**
	N	211	60	128
Fluconazole 72	MIC 50	8.0	8.0	8.0
	MIC 90	16	16	16
	Range	0.125 – 512	1 – 32	0.0625 - 32
	Geometric mean	**7.90**	**6.35**	**6.41**
	95% CI Geometric mean	**7.08 – 8.82**	**5.19 – 7.77**	**5.64 – 7.28**
	N	212	60	128
Itraconazole	MIC 50	0.128	0.128	0.064
	MIC 90	0.256	0.256	0.128
	Range	0.004 – 1.024	0.032 – 0.256	0.004 – 2
	Geometric mean	0.082	0.099	0.064
	95% CI Geometric mean	0.073 – 0.093	0.083 – 0.116	0.055 – 0.075
	N	211	60	128
Ketoconazole	MIC 50	0.064	0.064	0.032
	MIC 90	0.128	0.256	0.064
	Range	0.004 – 1.024	0.016 – 0.256	0.004 – 0.256
	Geometric mean	0.061	0.065	0.033
	95% CI Geometric mean	**0.055 – 0.068**	**0.054 – 0.079**	**0.029 – 0.038**
	N	212	60	128
Posaconazole	MIC 50	0.128	0.128	0.128
	MIC 90	0.256	0.256	0.256
	Range	0.008 – 0.512	0.064 – 0.512	0.008 – 0.512
	Geometric mean	0.113	0.119	0.110
	95% CI Geometric mean	0.103 – 0.125	0.100 – 0.142	0.097 – 0.125
	N	213	59	128
Voriconazole	MIC 50	0.064	0.064	0.064
	MIC 90	0.128	0.128	0.128
	Range	0.004 – 0.512	0.016 – 0.256	0.004 – 0.256
	Geometric mean	0.054	0.063	0.046
	95% CI Geometric mean	0.049 – 0.060	0.052 – 0.076	0.040 – 0.052
	N	211	60	129

Table 5.5 Effect of HIV status on geometric mean MIC

Drug	Status	Coefficient	P Value
Amphotericin B	HIV Infected	1.0	-
	HIV Uninfected	**0.87**	**0.028**
	Unknown	**0.74**	**<0.0001**
Flucytosine	HIV Infected	1.0	-
	HIV Uninfected	**0.70**	**<0.0001**
	Unknown	**0.61**	**<0.0001**
Fluconazole	HIV Infected	1.0	-
	HIV Uninfected	0.80	0.06
	Unknown	**0.81**	**0.0178**
Itraconazole	HIV Infected	1.0	-
	HIV Uninfected	1.19	0.16
	Unknown	**0.78**	**0.01**
Ketoconazole	HIV Infected	1.0	-
	HIV Uninfected	1.075	0.52
	Unknown	**0.55**	**<0.0001**
Posaconazole	HIV Infected	1.0	-
	HIV Uninfected	1.05	0.64
	Unknown	0.97	0.70
Voriconazole	HIV Infected	1.0	-
	HIV Uninfected	1.17	0.15
	Unknown	0.85	0.052

There were statistically significant differences between groups in geometric mean MICs when compared to isolates from known HIV infected sources. Clinical isolates from known HIV infected patients were less susceptible to antifungal agents than isolates from HIV uninfected patients or from patients of unknown HIV infection status. This was independent of drug class, holding true for amphotericin B and flucytosine for both the HIV uninfected and unknown status groups, and in addition for fluconazole, itraconazole and ketoconazole for isolates of unknown HIV serostatus source. In addition, the difference in fluconazole MICs between HIV infected and HIV uninfected patients was close to statistical significance ($p = 0.057$), but must be interpreted in the light of multiple tests having been performed.

5.4.3 Effect of year

I determined the effect of year on 72 hour MIC over the 13 year period on the whole group, and then by HIV status. Figures 5.2 to 5.4 are scatter plots illustrating the time trend in $\log_2$ MICs for amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole, posaconazole and voriconazole respectively for the whole group of strains. The blue lines represent the linear regression lines and the red lines are lowess (locally weighted scatter plot smoother) lines.

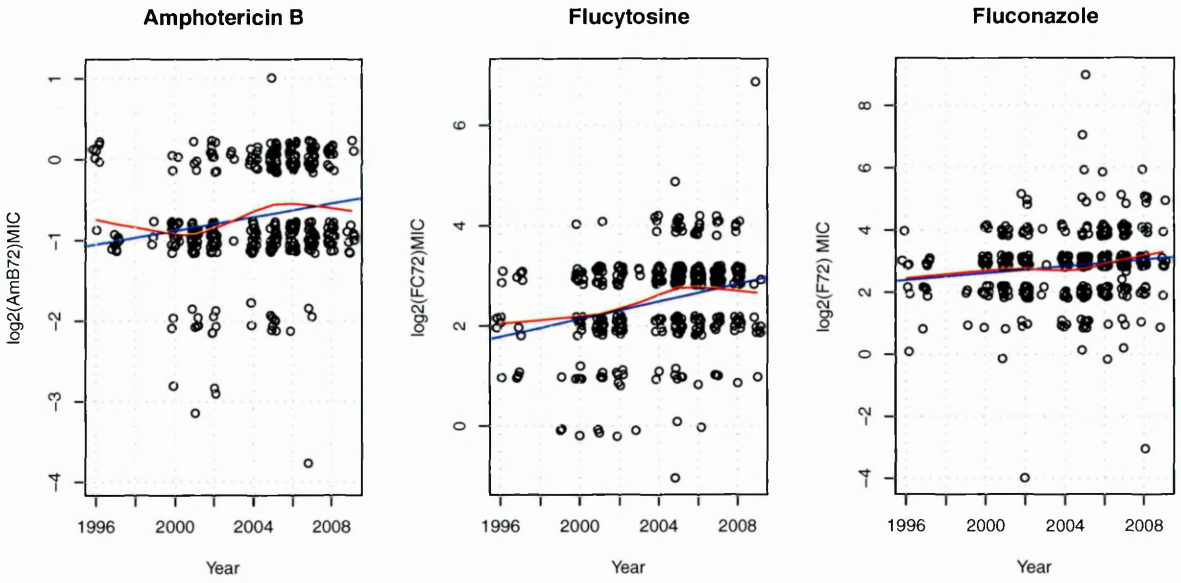


Figure 5.2 Trends in amphotericin B, flucytosine and fluconazole MICs over the 13 year study period. Red line = lowess line, blue line = regression line.

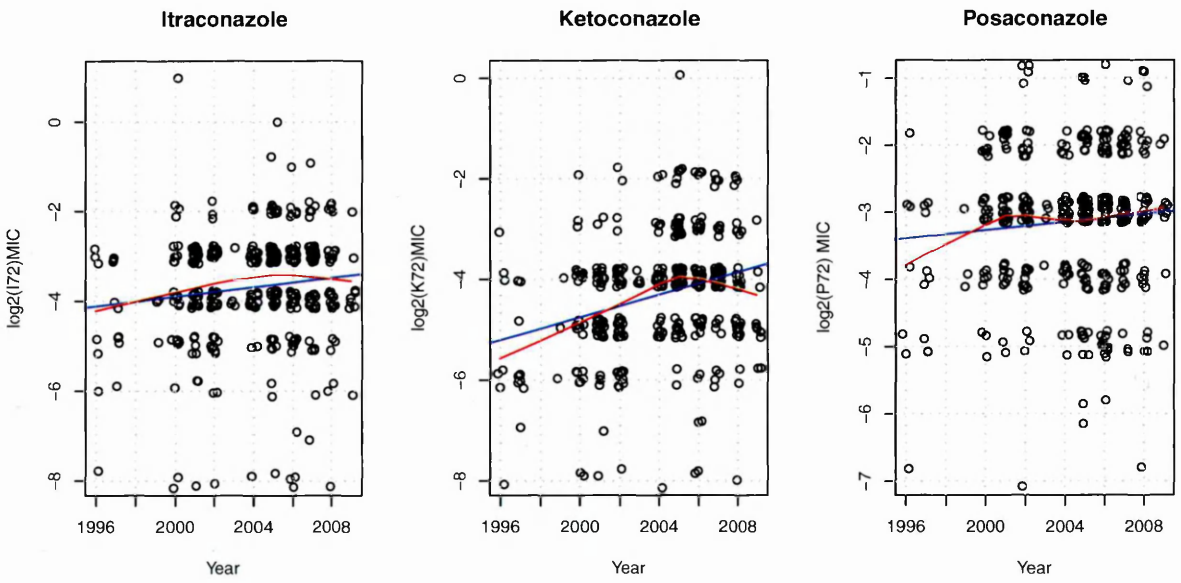


Figure 5.3 Trends in itraconazole, ketoconazole and posaconazole MICs over the 13 year study period. Red line = lowess line, blue line = regression line.

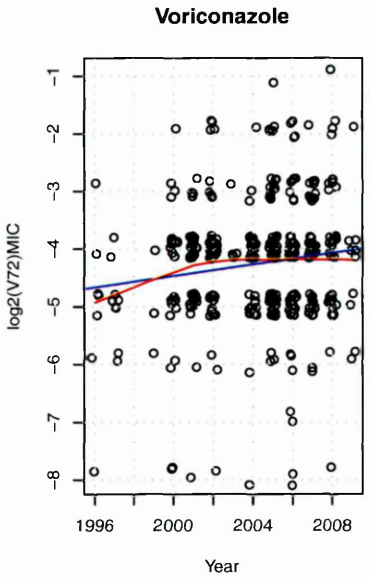


Figure 5.4 Trends in voriconazole MIC over the 13 year study period. Red line =lowess line, blue line – regression line.

Linear regression was used to investigate the relationship between time (years) and MICs. The coefficients describing changes in MICs for the antifungals are shown in table 5.6 below.

Table 5.6

Change in MIC of 7 antifungal drugs over time

	Coefficient of yearly change in MIC	Coefficient of change in MIC 1996 – 2009		P value
Antifungal	**Value**	**Value**	**95% CI**	
Amphotericin B	1.03	1.46	1.21 – 1.76	<0.001
Flucytosine	1.06	2.20	1.70 – 2.85	<0.001
Fluconazole	1.04	1.63	1.16 – 2.28	<0.001
Itraconazole	1.04	1.61	1.11 – 2.33	0.013
Ketoconazole	1.08	2.74	1.94 – 3.86	<0.001
Posaconazole	1.02	1.31	0.96 – 1.79	0.08
Voriconazole	1.04	1.57	1.13 – 2.17	0.007

With the exception of posaconazole the MICs for all the drugs tested increased over the 13 year period. The greatest changes were seen for flucytosine and ketoconazole, where there was a doubling of MIC over the time period. The posaconazole MIC appeared to increase but did not reach conventional levels of statistical significance. The linear model assumes that the change in $\log_2$ MICs can be described with a straight line. However, for amphotericin B, flucytosine, ketoconazole and posaconazole the scatter plots suggest that the relationship may not be linear, since lowess lines do not closely follow the regression lines (figures 5.2 and 5.3). Thus I also modelled the relationship using a natural cubic spline with 5 degrees of freedom and used a global F-test to assess whether there was an effect of year on MIC levels. Changes in MIC by year remained statistically significant for amphotericin B ($p<0.001$), flucytosine ($p<0.001$),

ketoconazole ($p<0.001$) and were also significant for posaconazole ($p<0.013$). As explained before, the estimated association cannot easily be quantified.

5.4.4 Effect of HIV serostatus

I then examined the effect of HIV serostatus on the change in MIC over time. The HIV infected group contained isolates from the years 2004 – 2009, the HIV uninfected isolates from 1996 to 2009, and the unknown HIV serostatus (A-strain) group from 1996 – 2002. I assumed all of the unknown HIV serostatus isolates were in fact HIV positive isolates, and pooled them with the definite HIV isolates to create a group of probable or definite HIV related isolates. I believed this to be reasonable since I was confident that 1) I had identified all HIV negative isolates and 2) through cross referencing by patient name knew that the HIV negative isolates were not duplicated in the A-strain group Moreover, it is much more probable that an isolate is from an HIV infected patient than not and thus that A-strain isolates are from HIV positive patients. The advantage of this grouping was that I now had HIV and non-HIV related isolates spanning the same time-period, 1996 – 2009. The results of the analysis are shown in Table 5.7 below.

Table 5.7

Linear time trend after adjusting for HIV serostatus

Antifungal	Variable	Coefficient	95% CI	P value
Amphotericin B	HIV positive	1.068	0.95 – 1.21	0.29
	Year	1.03	1.02 – 1.05	<0.001
Flucytosine	HIV positive	1.29	1.09 – 1.52	0.003
	Year	1.07	1.05 – 1.09	<0.001
Fluconazole	HIV positive	1.22	0.98 – 1.51	0.07
	Year	1.04	1.02 – 1.07	0.002
Itraconazole	HIV positive	0.80	0.63 – 1.01	0.06
	Year	1.03	1.00 – 1.06	0.03
Ketoconazole	HIV positive	0.82	0.66 – 1.02	0.08
	Year	1.08	1.05 – 1.11	<0.001
Posaconazole	HIV positive	0.97	0.79 – 1.18	0.74
	Year	1.02	0.99 – 1.05	0.10
Voriconazole	HIV positive	0.84	0.68 – 1.03	0.09
	Year	1.03	1.01 – 1.06	0.02

The analysis shows that after adjusting for year the study characteristic (HIV positive or not) is not statistically significantly associated with a difference in MIC for amphotericin B or posaconazole (p=0.29 and p=0.74 respectively), but is associated with the MIC of flucytosine p = 0.003). The MICs of flucytosine were higher in general for HIV infected patients compared with HIV uninfected patients. For fluconazole, itraconazole, ketoconazole and voriconazole I did not demonstrate an influence of HIV serostatus on

MIC after adjusting for year, although the p values approached conventional levels of significance (p = 0.07, 0.06, 0.08 and 0.09 respectively). The MICs of all drugs with the exception of posaconazole increased by year, independent of the HIV serostatus of the isolate source (table 5.7).

This analysis makes the assumption that the effect of year on MIC is the same for isolates whether or not they are from HIV infected patients. To investigate whether there is in fact an interaction between HIV status and time for antifungal MICs (i.e. the effect of year is different depending upon the HIV serostatus) I modelled an interaction between time and HIV serostatus. Scatter plots for the MICs of each antifungal drug by year according to HIV status are shown in figures 5.5 to 5.11 below.

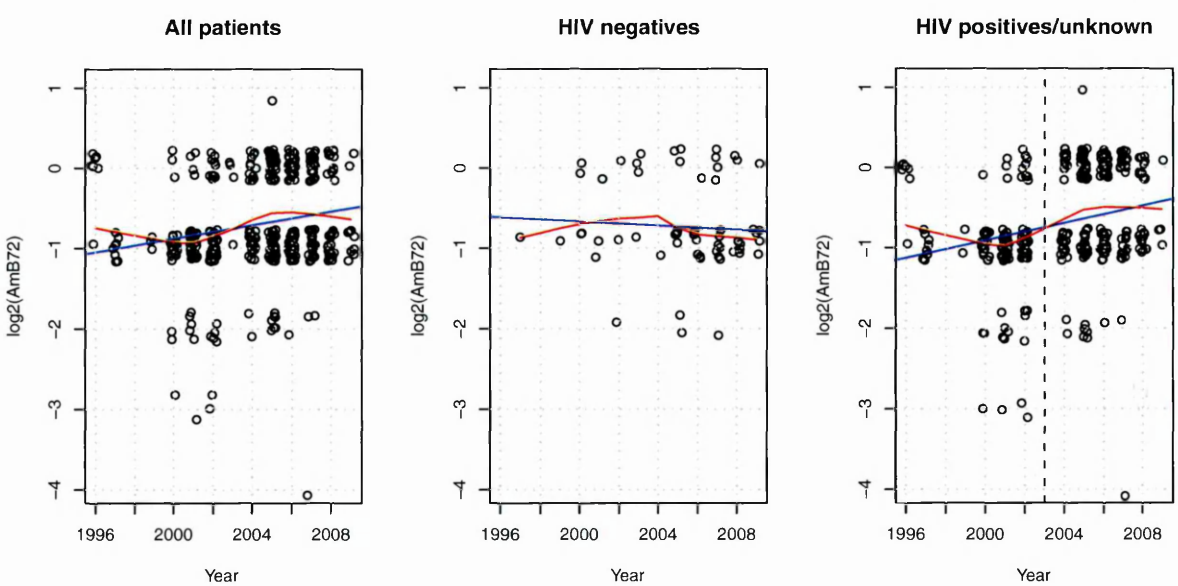


Figure 5.5 Time trend by year in MICs of amphotericin B for the whole strain collection, and divided by HIV status.

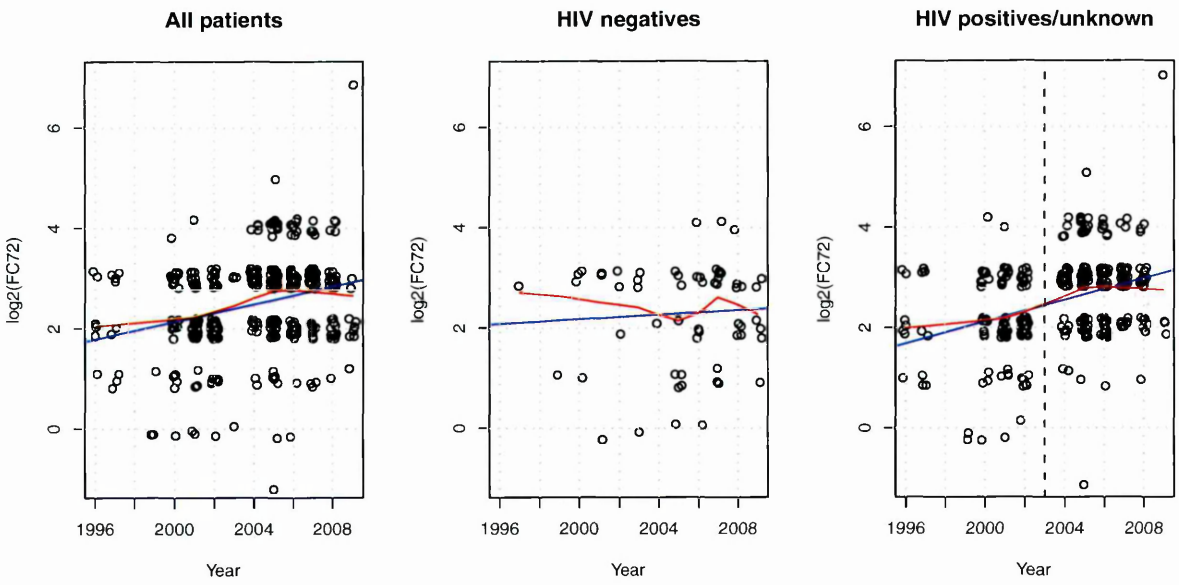


Figure 5.6 Time trend by year in MICs of flucytosine for the whole strain collection, and divided by HIV status.

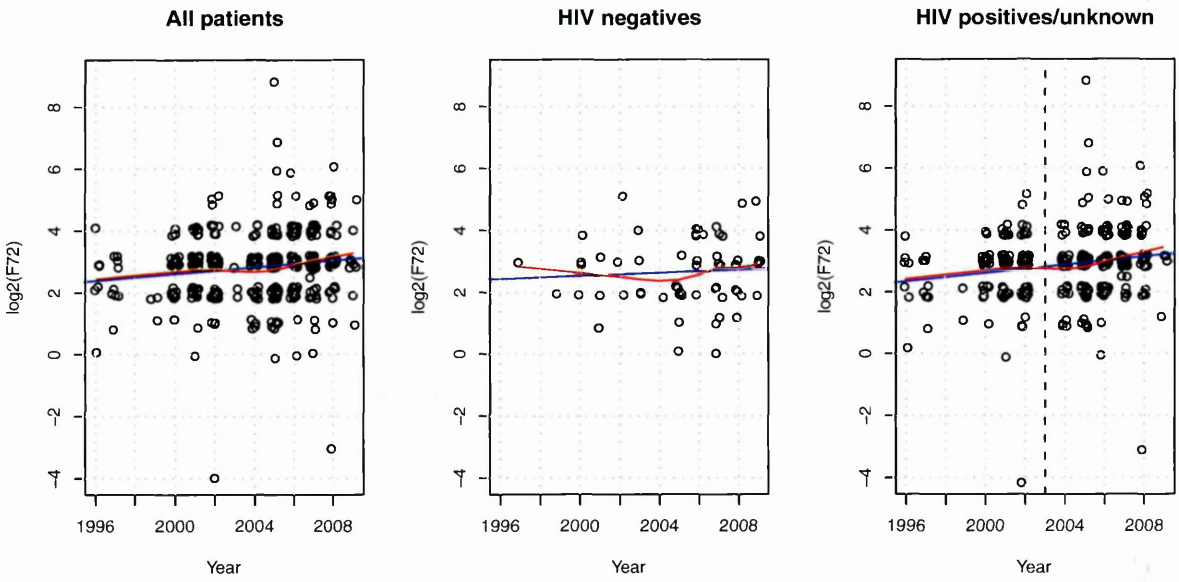


Figure 5.7 Time trend by year in MICs of fluconazole for the whole strain collection, and divided by HIV status.

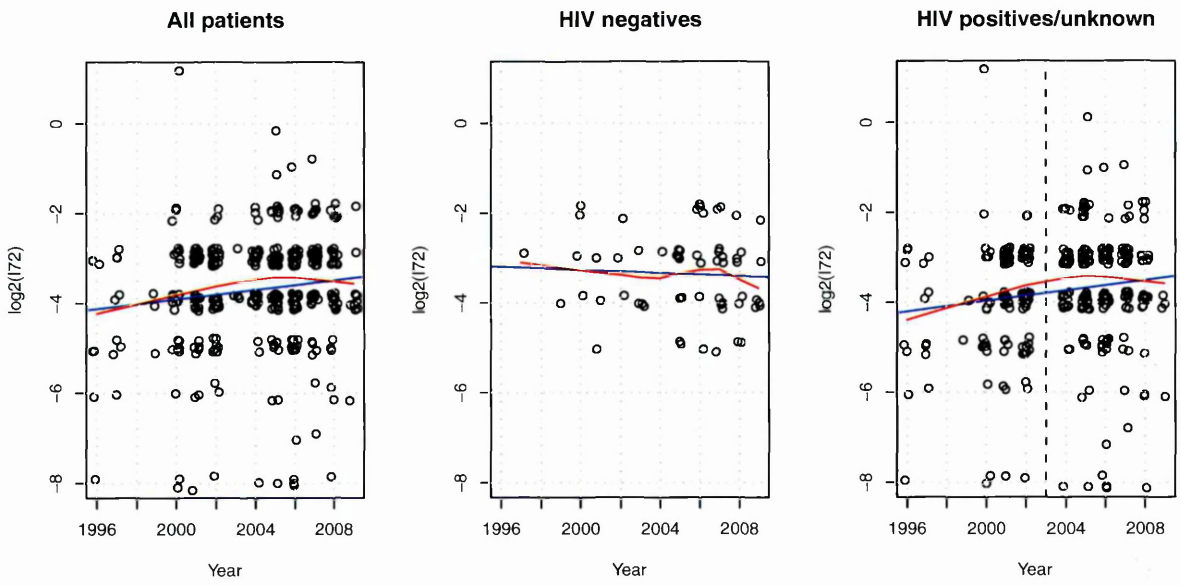


Figure 5.8 Time trend by year in MICs of itraconazole for the whole strain collection, and divided by HIV status.

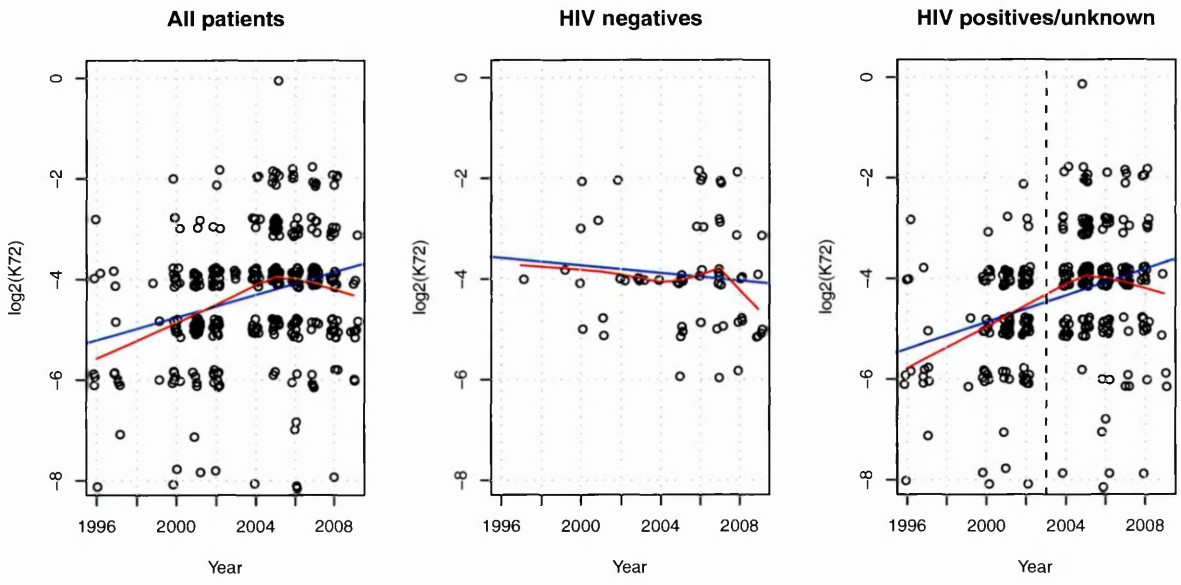


Figure 5.9 Time trend by year in MICs of ketoconazole for the whole strain collection, and divided by HIV status..

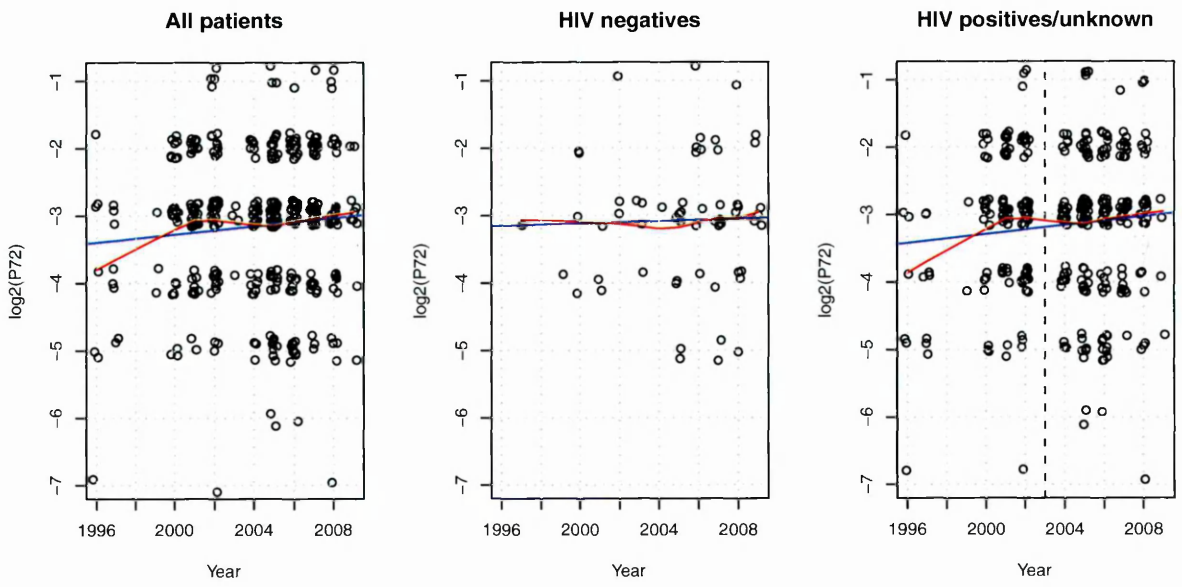


Figure 5.10 Time trend by year in MICs of posaconazole for the whole strain collection, and divided by HIV status.

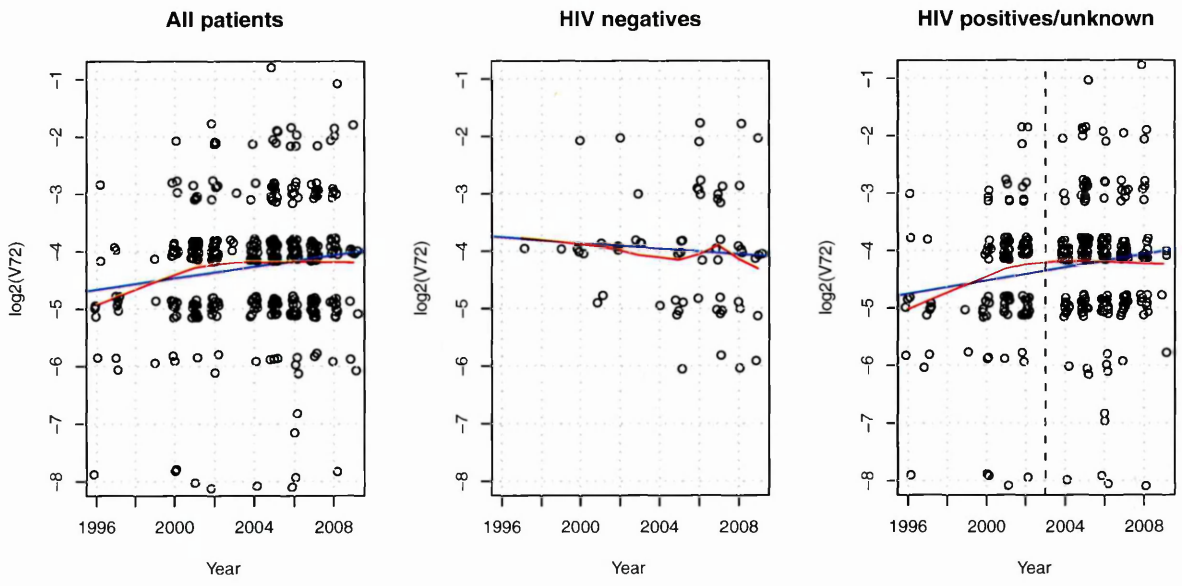


Figure 5.11 Time trend by year in MICs of voriconazole for the whole strain collection, and divided by HIV status.

The blue line represents the regression line of the linear model, the red line is a lowess (smoother) line, and the dotted line divides the A-strain (probably HIV associated) from the BK (definitely HIV associated) strains. While the plots are suggestive that the effect of year on antifungal MIC may be different according to HIV serostatus for amphotericin B, flucytosine, itraconazole, ketoconazole and voriconazole, the modelling suggests that the effect is different only for amphotericin B (p=0.03), flucytosine (p=0.04), and ketoconazole (p=0.002). The p values for the other antifungals were: fluconazole p=0.44, itraconazole p=0.21, posaconazole p=0.63 and voriconazole p=0.17.

5.4.5 Effect of Species

Species identification was performed in 366 isolates using *URA5* PCR-RFLP as described in Chapter Two. 350 isolates were *Cryptococcus grubii* (all molecular type VN1). 16 isolates (4.3%) were *C. gattii* (15 molecular type VG1 and 1 molecular type VG2). The results of antifungal testing according to species are shown in Table 5.8. The geometric mean 72 hour MICs of amphotericin and flucytosine were lower for *C. gattii* compared with *C. grubii* (0.632 mg/L versus 0.49 mg/L, p=0.05 and 6.09 mg/L versus 2.46mg/L, p=0.0002 respectively). The MIC_{90}s for each species were the same for each drug (1.024 mg/L for amphotericin; 16mg/ml for flucytosine). No significant differences in geometric mean MIC were detected for the azole drugs.

I examined the linear trends with time of MIC of amphotericin B, fluconazole and flucytosine by species using linear regression as described previously. The plots are show in figures 5.12 – 5.14 below.

Table 5.8 MICs by Species, ug/mL

Antifungal		Species *C. grubii* (n – 350)	*C. gattii* (n = 16)	P value (t test)
Amphotericin B	MIC 50	0.512	0.512.	
	MIC 90	1.024	1.024	
	Range	0.064 – 2.048	0.256 – 1.024	
	Geometric mean	**0.632**	**0.490**	**0.05**
	95% CI Geometric mean	**0.604 – 0.661**	**0.381 – 0.630**	
	N	350	16	
Flucytosine	MIC 50	8	2	
	MIC 90	8	8	
	Range	0.48 - 128	0.96 - 8	
	Geometric mean	**6.09**	**2.46**	**0.0002**
	95% CI Geometric mean	**5.72 – 6.48**	**1.64 – 3.69**	
	N	347	16	
Fluconazole	MIC 50	8.0	4	
	MIC 90	16	16	
	Range	0.0625 – 512	2 - 16	
	Geometric mean	7.33	5.42	0.12
	95% CI Geometric mean	6.74 – 7.98	3.70 – 7.93	
	N	348	16	
Itraconazole	MIC 50	0.064	0.096	
	MIC 90	0.128	0.256	
	Range	0.004 – 2.0	0.032 – 0.256	
	Geometric mean	0.078	0.095	0.29
	95% CI Geometric mean	0.071 – 0.086	0.066 – 0.135	
	N	347	60	
Ketoconazole	MIC 50	0.064	0.064	
	MIC 90	0.128	0.256	
	Range	0.004 – 1.024	0.016 – 0.256	
	Geometric mean	0.053	0.061	0.47
	95% CI Geometric mean	0.048 – 0.057	0.040 – 0.095	
	N	347	16	
Posaconazole	MIC 50	0.128	0.128	
	MIC 90	0.256	0.256	
	Range	0.008 – 0.512	0.032 – 0.512	
	Geometric mean	0.114	0.108	0.76
	95% CI Geometric mean	0.106 – 0.123	0.073 – 0.159	
	N	348	16	
Voriconazole	MIC 50	0.064	0.064	
	MIC 90	0.128	0.256	
	Range	0.004 – 0.512	0.032 – 0.256	
	Geometric mean	0.052	0.064	0.27
	95% CI Geometric mean	0.048 – 0.056	0.044 – 0.094	
	N	347	16	

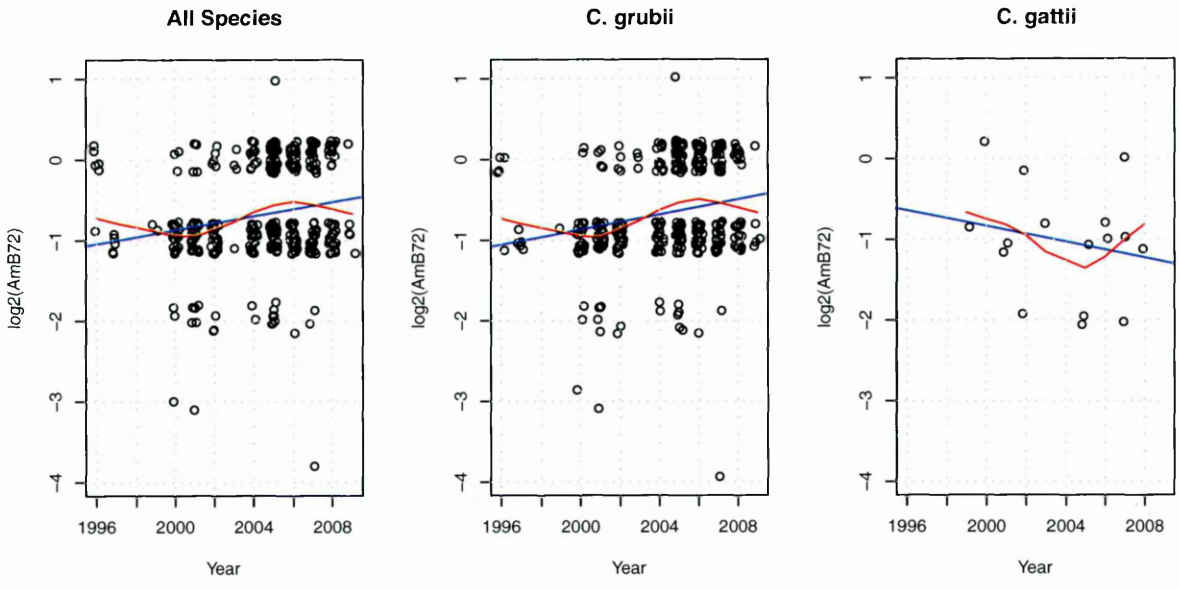


Figure 5.12 Year trends in amphotericin B MICs by *Cryptococcus* species.

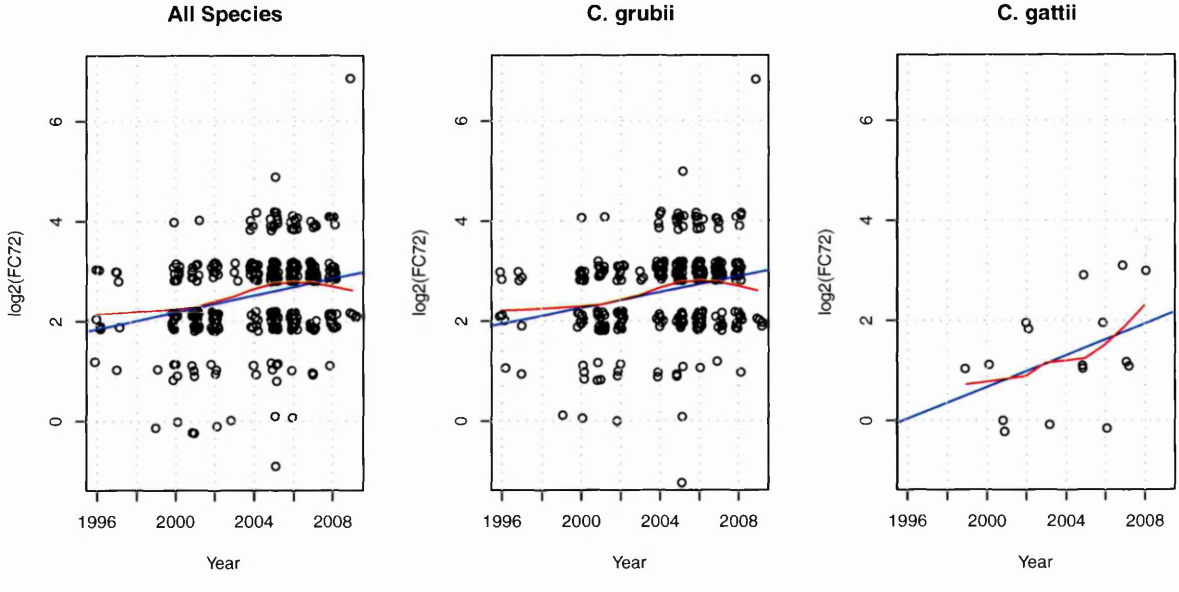


Figure 5.13 Year trends in flucytosine MICs by *Cryptococcus* species.

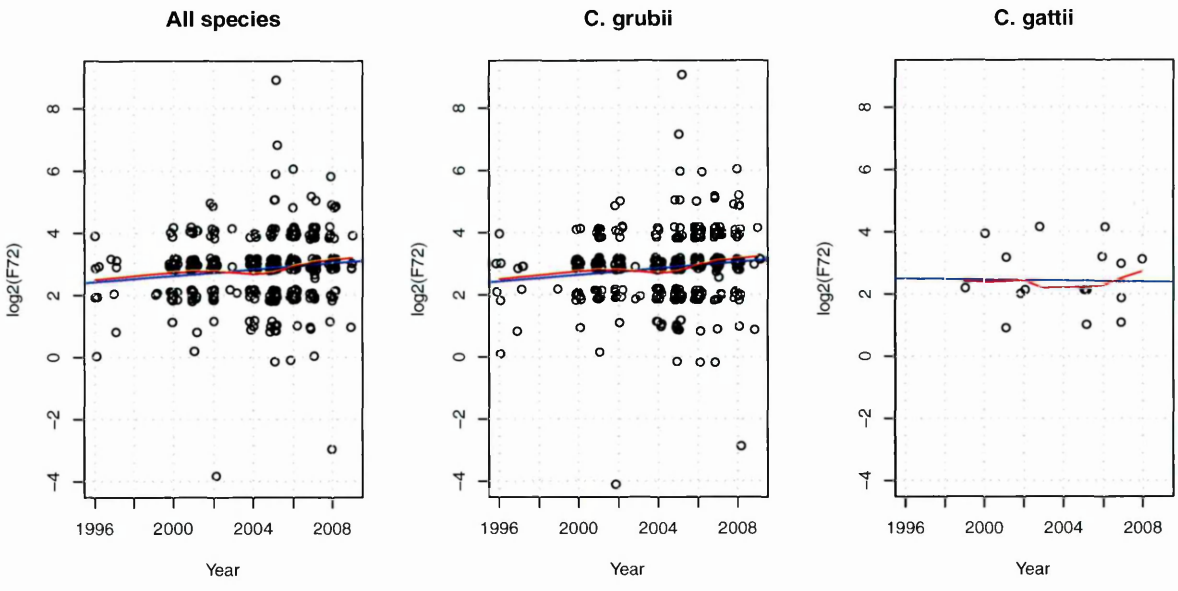


Figure 5.14 Year trends in fluconazole MICs by *Cryptococcus* species.

The analyses are limited by the small number of cases of *C. gattii*. As expected from the previous analysis, yearly trends in MIC were demonstrable in *C. grubii* but failed to reach statistical significance for *C. gattii* Table 5.9).

Table 5.9

Changes in Geometric mean MIC of amphotericin B, flucytosine and fluconazole over 13 years by *Cryptococcus* species.

Antifungal	Species					
	C. grubii			*C. gattii*		
	Change in geometric mean MIC over 13 years			Change in geometric mean MIC over 13 years		
	Coefficient	95% CI	P	Coefficient	95% CI	P
Amphotericin	1.52	1.24 – 1.87	<0.001	0.64	0.19 – 2.14	0.44
Flucytosine	2.03	1.55 – 2.67	<0.001	4.14	0.68 – 25.5	0.11
Fluconazole	1.59	1.08 – 2.34	0.018	0.93	0.14 – 6.00	0.93

5.4.6 Effect of AFLP clade on antifungal MICs

In Chapter 4, using AFLP, I showed that all cases of cryptococcal meningitis in Viet Nam due to *Cryptococcus grubii* are due to infection with 1 of 2 clades of *URA5* PCR-RFLP molecular group 1. I found that HIV uninfected patients were more likely to be infected with strains from Clade 1 than Clade 2 (odds ratio 5.93, 95% confidence interval 2.4 – 16.6, $p<0.0001$). I examined whether there were any differences in drug susceptibility according to infecting clade. The results are shown in table 5.10 below. There were no significant differences in antifungal susceptibility, other than a possible difference in the geometric mean MIC of posaconazole, which was slightly higher in Clade 2. However, there were no differences in MIC_{50} or MIC_{90}.

I examined the linear trends with time in MIC of amphotericin B, fluconazole and flucytosine by clade using linear regression. The plots are show in figures 5.15 – 5.17 below.

Table 5.10 MICs by *C. grubii* clade, ug/mL

Antifungal		Clade *Clade 1 (n = 67)*	*Clade 2 (n = 65)*	P value (t test)
Amphotericin B	MIC 50	1.024	0.512.	
	MIC 90	1.024	1.024	
	Range	0.256 – 2.048	0.064 – 1.024	
	Geometric mean	0.720	0.641	0.15
	95% CI Geometric mean	0.649 – 0.799	0.567 – 0.724	
	N	67	65	
Flucytosine	MIC 50	8	8	
	MIC 90	16	16	
	Range	2 – 16	2 – 16	
	Geometric mean	6.964	7.025	0.923
	95% CI Geometric mean	6.19 – 7.84	6.14 – 8.03	
	N	65	64	
Fluconazole	MIC 50	8.0	8	
	MIC 90	16	16	
	Range	1 - 128	0.125 - 64	
	Geometric mean	6.64	7.55	0.38
	95% CI Geometric mean	5.47 – 8.07	6.06 – 9.41	
	N	67	65	
Itraconazole	MIC 50	0128	0.064	
	MIC 90	0.256	0.256	
	Range	0.004 – 1.0	0.004 – 0.512	
	Geometric mean	0.088	0.068	0.13
	95% CI Geometric mean	0.072 - 0.106	0.047 – 0.089	
	N	66	64	
Ketoconazole	MIC 50	0.064	0.064	
	MIC 90	0.128	0.256	
	Range	0.004 – 1.024	0.004 – 0.256	
	Geometric mean	0.064	0.057	0.42
	95% CI Geometric mean	0.053 – 0.078	0.046 – 0.071	
	N	66	64	
Posaconazole	MIC 50	0.128	0.128	
	MIC 90	0.256	0.256	
	Range	0.032 – 0.512	0.008 – 0.512	
	Geometric mean	**0.123**	**0.093**	**0.036**
	95% CI Geometric mean	**0.105 – 0.144**	**0.076 – 0.114**	
	N	66	65	
Voriconazole	MIC 50	0.064	0.064	
	MIC 90	0.128	0.128	
	Range	0.004 – 0.512	0.004 – 0.512	
	Geometric mean	0.054	0.053	0.93
	95% CI Geometric mean	0.045 – 0.065	0.043 – 0.066	
	N	66	65	

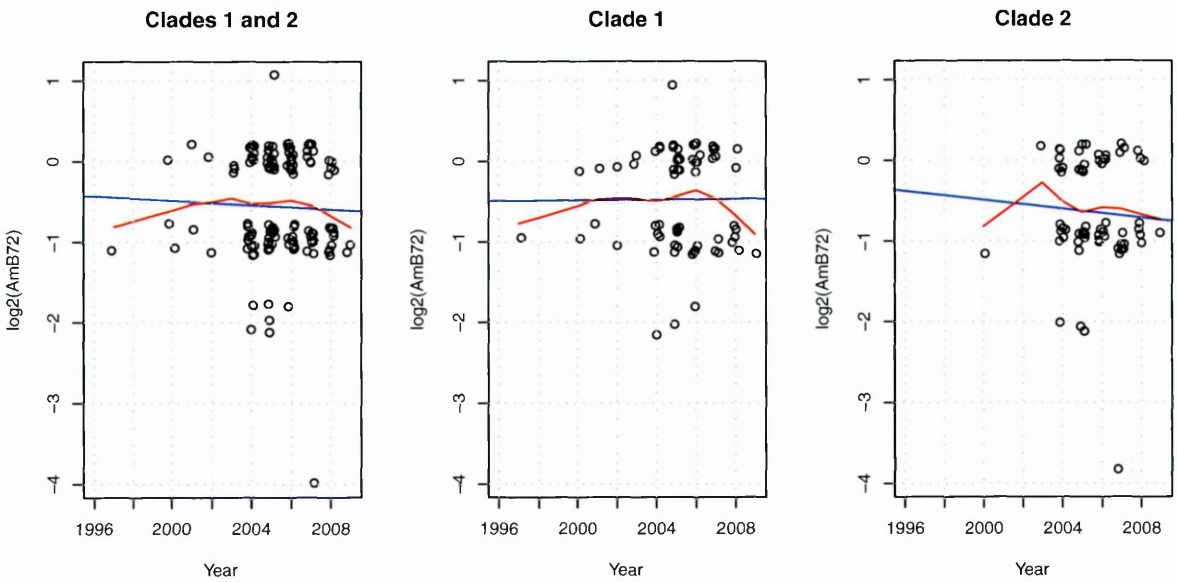


Figure 5.15 Year trends in amphotericin B MICs by *Cryptococcus grubii* clade.

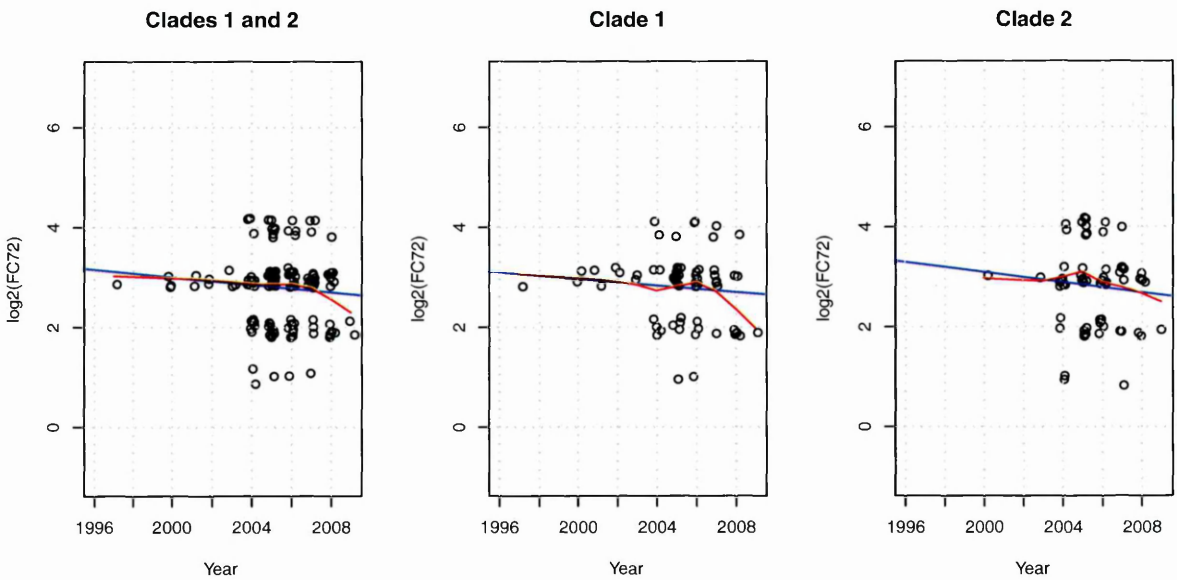


Figure 5.16 Year trends in flucytosine MICs by *C. grubii* clade.

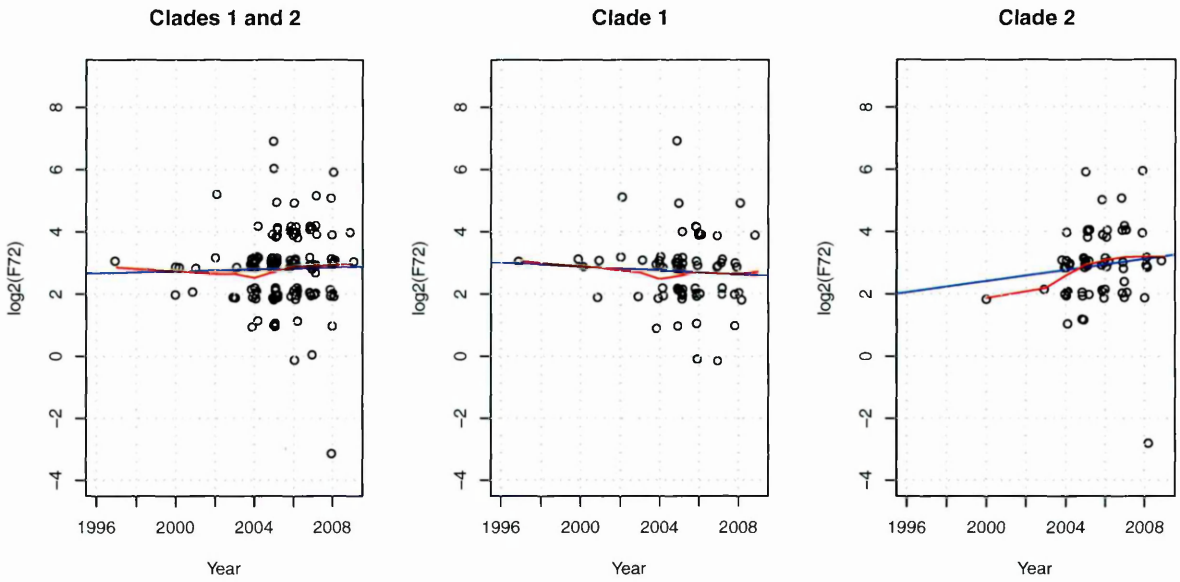


Figure 5.17 Year trends in fluconazole MICs by *C. grubii* clade.

There were no statistically significant changes in MIC over time detected using the linear model (table 5.11).

Table 5.11

Changes in Geometric mean MIC of amphotericin B, flucytosine and fluconazole over 13 years by *Cryptococcus grubii* Clade

Antifungal	Clade					
	Clade 1			Clade 2		
	Change in geometric mean MIC over 13 years			Change in geometric mean MIC over 13 years		
	Coefficient	95% CI	P	Coefficient	95% CI	P
Amphotericin	1.02	0.54 – 1.92	0.96	0.78	0.28 – 2.20	0.63
Flucytosine	0.74	0.36 – 1.52	0.41	0.63	020 – 1.97	0.42
Fluconazole	0.75	0.23 – 2.48	0.64	2.24	0.34 – 14.4	0.40

5.4.7 Correlation between susceptibility testing results at 48 and 72 hours

Cryptococcus neoformans is relatively slow growing compared with other yeast species such as *Candida*. While breakpoints have not been determined for *Cryptococcus spp*, the YeastOne® Sensititre® manufacturers recommend that MICs of antifungals for *Cryptococcus* are read at 72 hours. However, there is often evidence of growth in the test plates, with colour change and thus estimable MIC, at 48 hours. More rapid reporting of results would enable earlier intervention to alter therapy with potential benefit to patients. Therefore, I determined the correlation between MICs estimated at 48 and 72 hours for the *Cryptococcus* strains tested. Table 5.12 records the numbers and percentage of strains where there was a change between 48 hour and 72 hour MIC. It was not possible to estimate the MIC at 48 hours in all isolates (hence $N < 402$ for all antifungals). Figures 5.18 – 5.20 plot the 48 hour MIC results against the 72 hour MIC results for all the tested isolates. The black identity line is a visual aid with intercept of 0 and gradient of 1. The red line is the linear regression line.

Table 5.12

Change in 48 and 72 Hour MIC results for 7 antifungal drugs

Drug	No Change Number Per cent	Increased Number Per cent	Pearson Correlation
Amphotericin B	101	270	0.53
	27.2	72.8	
Flucytosine	146	223	0.72
	39.6	60.4	
Fluconazole	153	217	0.76
	41.4	58.6	
Itraconazole	108	259	0.78
	29.4	70.6	
Ketoconazole	175	195	0.8
	47.3	52.7	
Posaconazole	168	202	0.78
	45.4	54.6	
Voriconazole	167	200	0.77
	45.5	54.5	

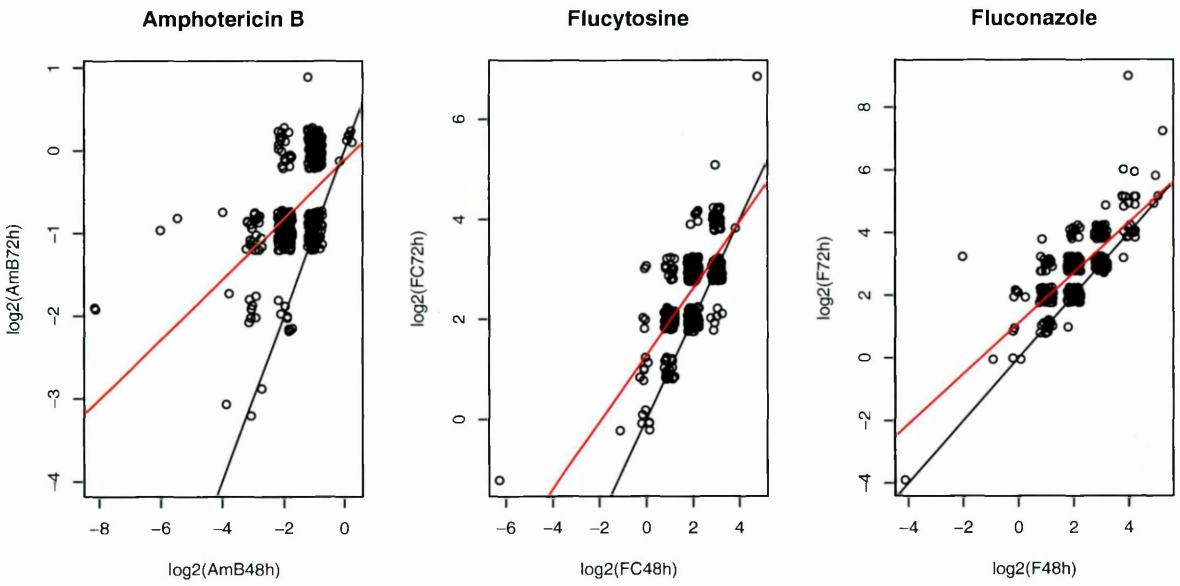


Figure 5.18.Plots of 72 hour log2 MIC against 48 hour log2 MIC for amphotericin, flucytosine and fluconazole.

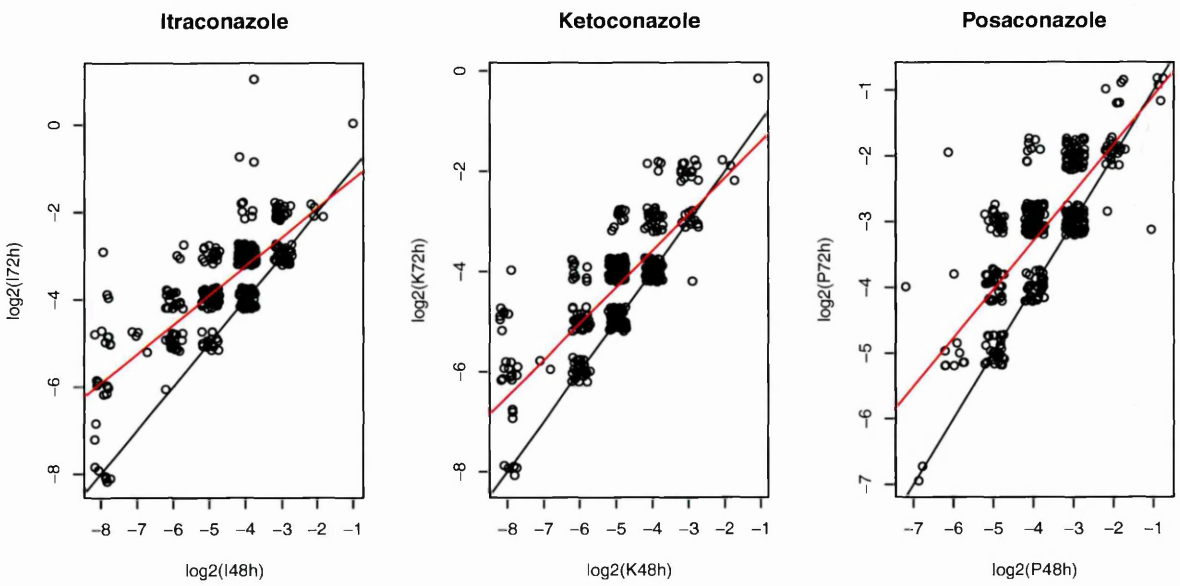


Figure 5.19 Plots of 72 hour log2 MIC against 48 hour log2 MIC for itraconazole, ketoconazole and posaconazole.

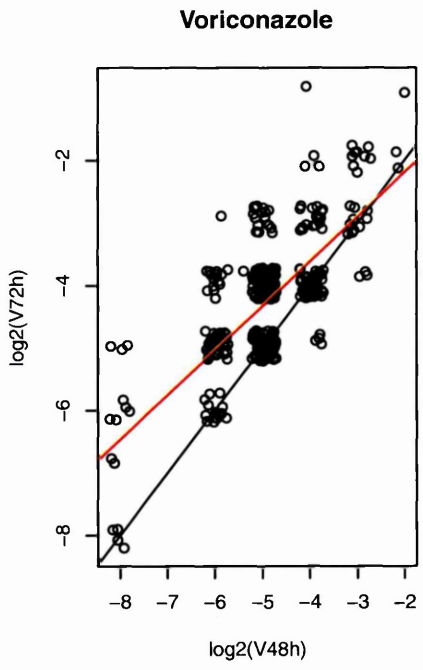


Figure 5.20.Plot of 72 hour log2 MIC against 48 hour log2 MIC for voriconazole.

I used linear regression to determine whether it was possible to correctly predict the 72 hour MIC from the 48 hour MIC. I looked at the absolute prediction rate, and the ability to predict the actual rate with an error of +/- one log2 MIC (i.e. one dilution of error). The results are displayed in Table 5.13.

Table 5.13

Number of 72 hour results correctly predicted from 48 hour result using the regression model

	Exact		+/- 1 dilution	
	Number		Number	
	%		%	
Drug	**Correct**	**Incorrect**	**Correct**	**Incorrect**
Amphotericin B	243	129	369	2
	65.3	34.7	99.5	0.5
Flucytosine	226	144	364	5
	61.1	38.9	98.6	1.4
Fluconazole	189	182	362	8
	50.9	49.1	97.8	2.2
Itraconazole	220	147	352	15
	59.9	40.1	95.9	4.1
Ketoconazole	203	167	358	12
	54.9	45.1	96.8	3.2
Posaconazole	216	154	367	3
	58.4	41.6	99.2	0.8
Voriconazole	197	170	358	9
	53.7	46.3	97.5	2.5

The 48 hour prediction of 72 hour MIC was good to within 1 dilution, with a maximum error rate of 4.1%.

5.4.8 Correlation between azole drug MICs

I examined the relationship between the MICs of different azole drugs. The scatter plots of the MICs of the 4 azole drugs fluconazole, itraconazole, posaconazole and voriconazole are shown in Figure 5.21. The numbers are the rank correlations between the MICs of the different drugs. The dotted identity lines are visual aids where the intercept is zero and the slope is 1.

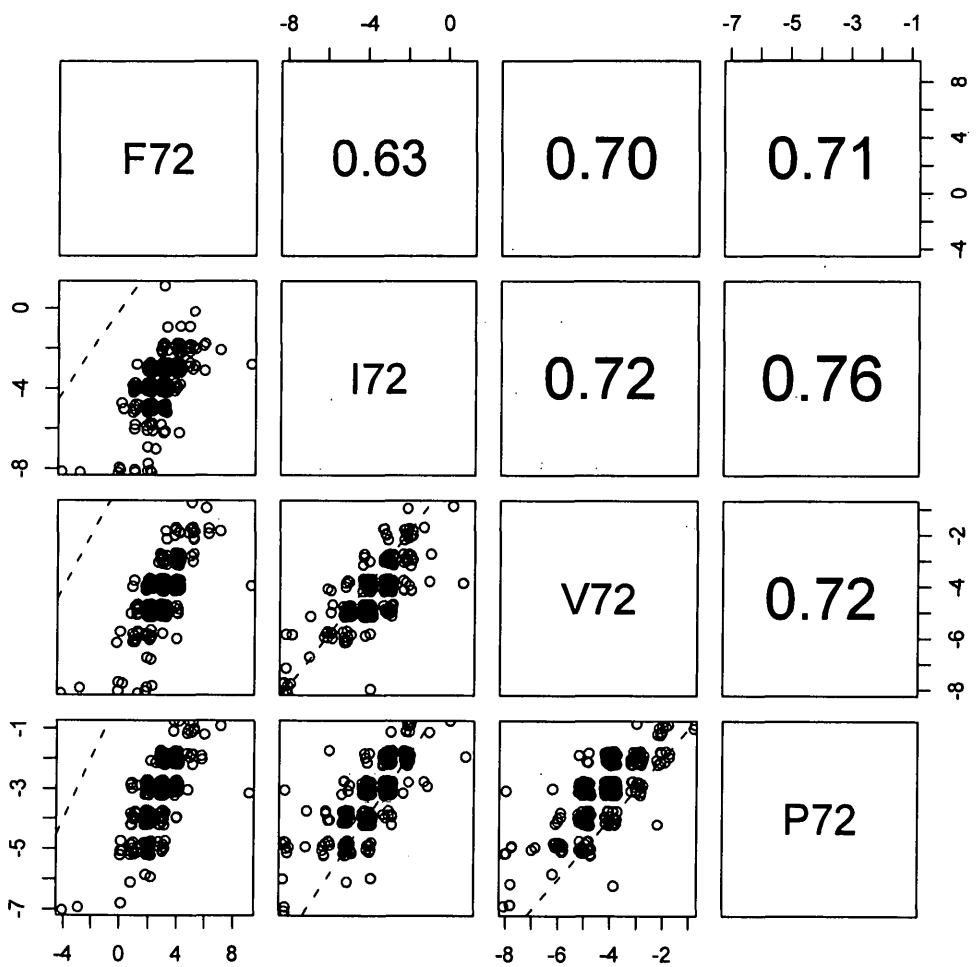


Figure 5.21 Scatterplots of azole MICs and rank correlations for 400 strains of *Cryptococcus* spp. F – fluconazole, I = Itraconazole, V = voriconazole, P = posaconazole.

The highest correlation was between posaconazole and itraconazole, and the lowest between fluconazole and itraconazole. I used linear regression to predict the 72 hour MICs of itraconazole, posaconazole and voriconazole from the 72 hour fluconazole MIC. The model predicted the actual MIC of itraconazole, posaconazole and voriconazole to +/- 1 dilution 90.2, 96.0 and 94.5% of the time respectively (table 5.14).

Table 5.14

Rates of Successful Prediction of Itraconazole, Posaconazole and Voriconazole MICs from the 72 hour fluconazole MIC

Drug	N	Exact	Within 1 dilution
		N	N
		%	%
Itraconazole	398	174	359
		43.7	90.2
Posaconazole	399	224	383
		56.1	96.0
Voriconazole	399	225	377
		56.4	94.5

Table 5.15 lists the errors in prediction made that were greater than one dilution from the actual result:

Table 5.15

Number of errors in prediction by dilution fold

Antifungal	N	2 dilution error	3 dilution error	4 dilution error	5 dilution error
Itraconazole	398	12	5	1	0
Posaconazole	399	1	1	0	0
Voriconazole	399	5	1	0	0

5.4.9 Influence of amphotericin B MIC on rate of clearance of yeast from CSF.

A total of 208 quantitative count measurements from 63 HIV-positive patients with cryptococcal meningitis were available; the median number of measurements per patients was 3 (range 1 to 8). Individual patient profiles of all measurements are displayed in figure 5.23.

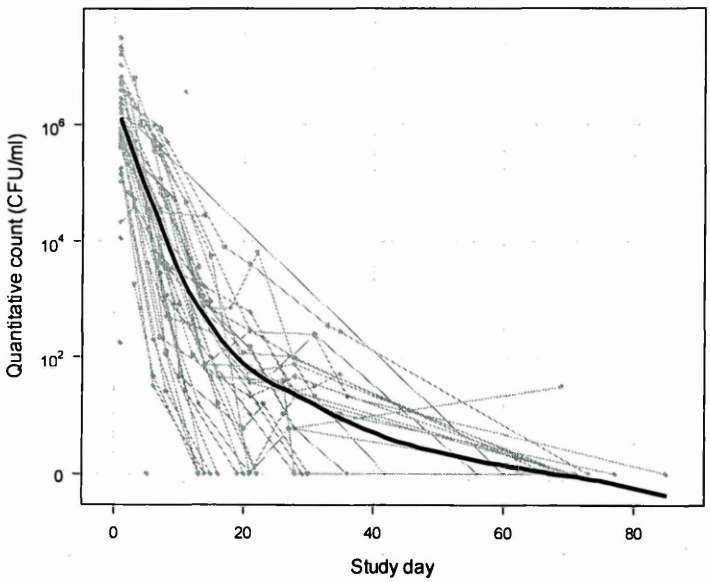


Figure 5.22 Quantitative counts from 63 HIV-positive patients with cryptococcal meningitis. Gray lines refer to individual patient data, the black line is a scatterplot smoother.

The basic model with an exponential decline determined a population intercept of 13.15 (95% CI [12.50, 13.81]) and slope -0.58 (95% CI [-0.67,-0.48]) on the logarithmic scale; the individual patient-specific deviations around these population parameters were estimated to have standard deviations of 2.11 and 0.26 for the intercept and slope, respectively. On the original scale, the slope corresponds to a decline of 44% per day (as exp(-0.58)=0.56). The extended model estimated an initial population slope of -0.73

(95% CI [-0.80, -0.66]) in the first 14 days and a decline in the population slope by 0.47 (95% CI [0.38, 0.76]) in the time thereafter. This equates to a decline in yeast count of 0.32 $\log_{10}$/day (0.73/$\log_e$10). The change in slope on day 14 was highly significantly different from zero ($p<0.001$) indicating a slower decline with increasing time of treatment. Figure 5.23 displays residuals versus time since treatment initiation suggesting that the extended model more adequately describes the data.

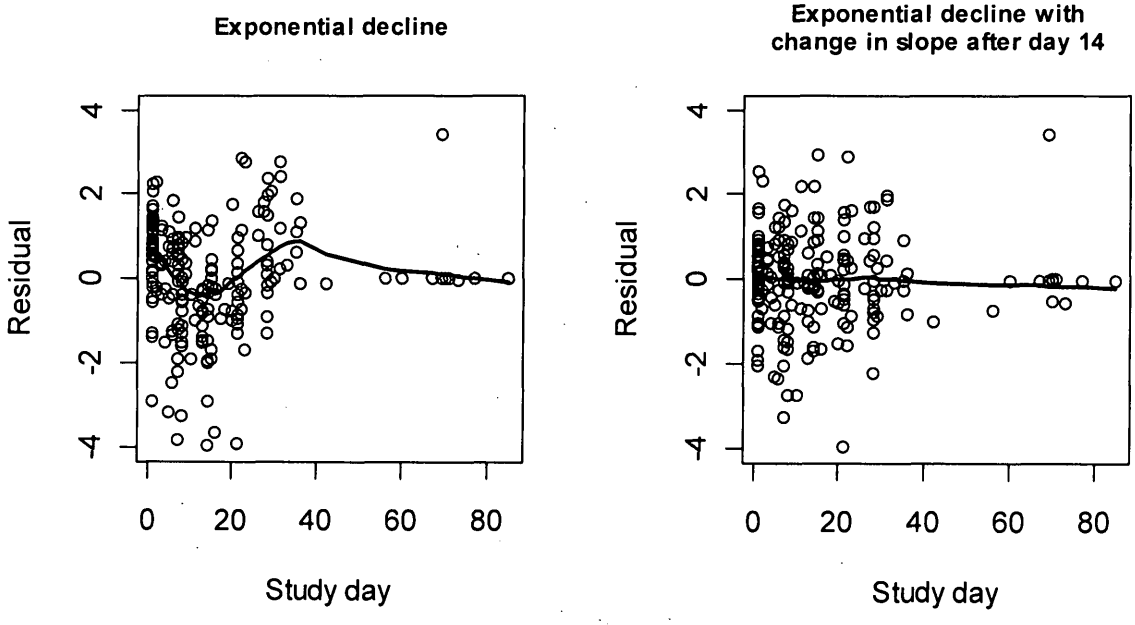


Figure 5.23 Residuals versus time since treatment initiation for the model with an exponential decline or the extended model, respectively. The black lines are (lowess) scatterplot smoothers.

Amphotericin B MIC data (72 hour result) were available for 60 (95%) of the 63 isolates. The range of MICs was narrow: 0.256 mg/L for one isolate (2%), 0.512 mg/L for 37 isolates (62%) and 1.024 mg/L for 22 (37%) isolates. Plots of quantitative counts over time stratified by amphotericin B MIC are displayed in figure 5.24. The extended model which postulated a linear effect of the covariate log2-amphotericin B MIC (72

hour result) on the initial quantitative count, the initial slope and the change in slope after day 14, respectively, did not significantly improve the model without this covariate (p[likelihood ratio test]=0.53); the effect of a 2-fold increase of the amphotericin B MIC on the initial slope was estimated as -0.09 (95% CI [-0.21,0.04], p=0.19). Similarly, the 48 hour amphotericin B MIC did not significantly affect the quantitative counts (p[likelihood ratio test]=0.36).

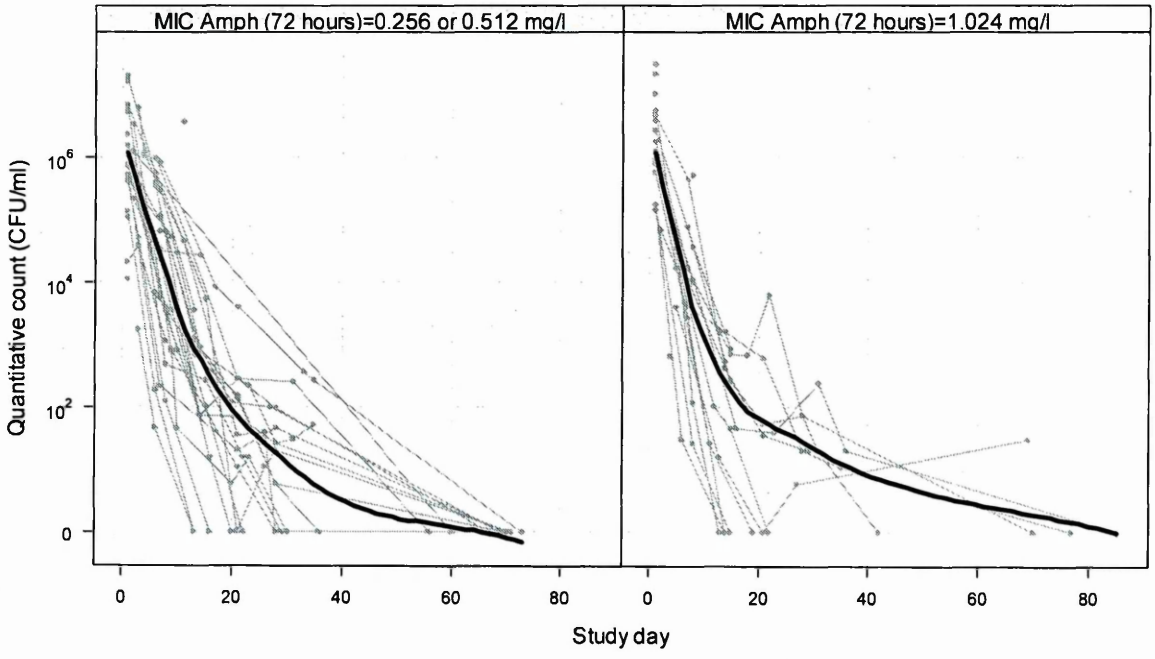


Figure 5.24 Quantitative counts by amphotericin B MIC. Gray lines refer to individual patient data, the black line is a scatterplot smoother.

Fluconazole MIC data (72 hour result) were available for 60 (95%) of the 63 isolates. The MIC was 2 mg/L for 4 isolates (7%), 4 mg/L for 13 isolates (22%), 8 mg/L for 27 isolates (45%), 16 mg/L for 13 isolates (22%) and between 32 and 512 mg/L for 3 further isolates (5%). Plots of quantitative counts over time stratified by fluconazole MIC are displayed in figure 5.25.

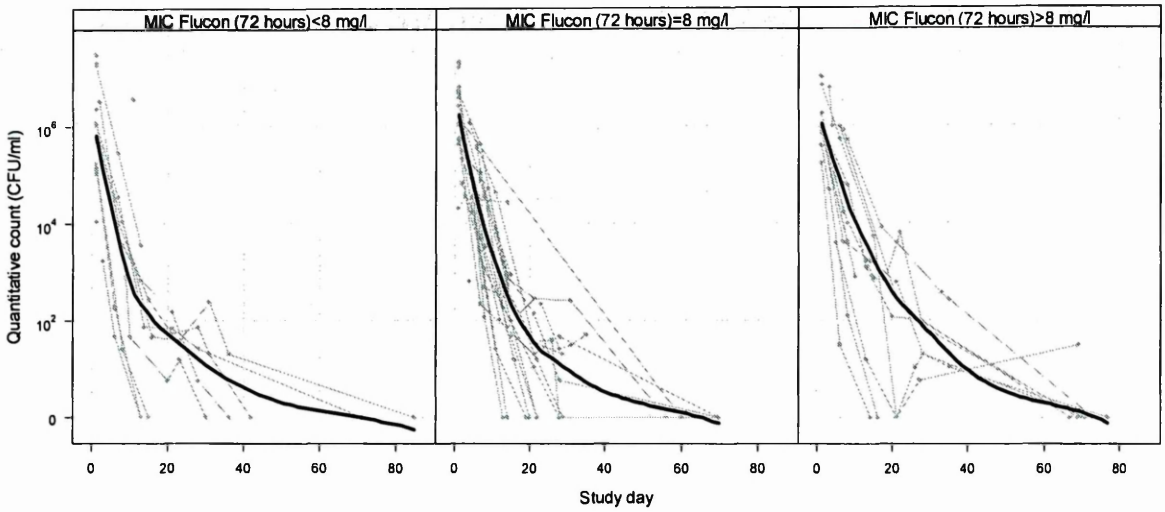


Figure 5.25. Quantitative counts by fluconazole MIC. Grey lines refer to individual patient data, the black line is a scatterplot smoother.

The extended model which postulated a linear effect of the covariate log2-fluconazole MIC (72 hour result) on the initial quantitative count, the initial slope and the change in slope after day 14, respectively, did not significantly improve the model without this covariate (p[likelihood ratio test]=0.11); the effect of a 2-fold increase of the fluconazole MIC on the initial slope was estimated as -0.03 (95% CI [-0.10,0.03], p=0.40). Similarly, the 48 hour fluconazole MIC did not significantly affect the quantitative counts (p[likelihood ratio test]=0.09); the trend towards significance was mainly due to a trend that the intercept (corresponding to the true log-quantitative count at treatment initiation) was estimated to increase by 0.44 (95% CI [-0.12,1.00], p=0.13) per 2-fold MIC increase.

The joint model for longitudinal counts and survival estimated a very similar population slope and intercept as the longitudinal model alone: estimates were 13.37 (95% CI [12.70, 14.03]) for the population intercept and -0.61 (95% CI [-0.73,-0.49]) for the slope. The Weibull model indicated a decreasing hazard of death with time with a shape parameter of 0.60 (95% CI [0.35,0.86]). There was no evidence that the rate of the quantitative count decline was associated with survival (HR=0.98; 95% CI [0.67,1.43] per +0.1 more shallow slope, p=0.91). However, there was a strong trend that higher true initial quantitative counts were related with a worse survival (HR=4.27; 95% CI [0.98,18.60] per ten-fold increase; p=0.05).

5.4.10 Correlation with Clinical Outcome

I used the Cox regression model to examine the effect of antifungal susceptibility on survival in the patients described in chapter 3. All patients received treatment with amphotericin B (1mg/kg/day) for the first 4 weeks of therapy, followed by treatment with fluconazole 400mg/day. Initially I performed univariate analysis looking at both 48 hour and 72 hour MIC estimations and the hazard of death at 70 days. I then corrected for fungal load, estimated by the CSF cryptococcal antigen titre and the viable yeast count, and performed a multiple Cox regression analysis including the amphotericin B and fluconazole MICs, and the CSF cryptococcal antigen titre. The results are shown in table 5.16. Results are only presented for amphotericin B (AmB), fluconazole (F) and flucytosine (FC). No patients received treatment with flucytosine.

I found no alteration in the hazard for death at 70 days according to MICs of amphotericin B, flucytosine or fluconazole, whether estimated at 48 hours or 72 hours. Correcting for fungal burden at baseline, estimated either by log10 quantitative count or log2 CSF cryptococcal antigen, had no effect, nor did correcting for fluconazole or amphotericin B MIC. No association was found between MICs of posaconazole, itraconazole, ketoconazole or voriconazole and hazard for death.

Table 5.16 Cox 70 day survival analysis according to antifungal MICs Univariate analysis for 48 and 72 hour MIC estimations

	MIC Result	Hazard Ratio	CI	P (Wald Test)	N
Antifungal	**AmB 48**	1.23	0.67 – 2.25	0.5	86
	AmB 72	1.0	0.52 – 1.9	0.994	96
	FC48	0.93	0.63 – 1.38	0.72	87
	FC72	1.12	0.76 -1.64	0.57	95
	F48	1.07	0.75 – 1.52	0.71	87
	F72	0.98	0.72 – 1.32	0.88	96
Analysis adjusted for fungal burden using CSF cryptococcal antigen titre.					
Antifungal	**AmB 48**	1.09	0.60 – 1.98	0.78	83
	AmB 72	1.1	0.56 – 2.16	0.78	93
	FC48	0.96	0.64 – 1.43	0.83	84
	FC72	1.02	0.71 – 1.48	0.91	92
	F48	1.02	0.71 – 1.46	0.92	84
	F72	0.92	0.65 – 1.30	0.63	93
Analysis adjusted for fungal burden using CSF cryptococcal viable count.					
Antifungal	**AmB 48**	1.24	0.64 – 2.4	0.53	52
	AmB 72	0.87	0.37 – 2.07	0.76	55
	FC48	0.75	0.46 – 1.22	0.25	52
	FC72	0.71	0.41 – 1.19	0.19	54
	F48	0.94	0.60 – 1.47	0.77	52
	F72	0.81	0.52 – 1.27	0.36	55
Analysis including both amphotericin B and fluconazole MICs at 48 or 72 hours, respectively, adjusted for CSF cryptococcal antigen titre					
Antifungal	**AmB48**	1.09	0.60 – 1.98	0.77	83
	F48	1.02	0.71 – 1.47	0.91	
	AmB72	1.12	0.57 – 2.21	0.73	93
	F72	0.91	0.64 – 1.29	0.60	

The Cox regression model was used to analyse the effect of antifungal susceptibility on early death, defined as occurring within 7, 14 or 28 days. I examined only amphotericin B susceptibility, since this was the only treatment received by the patients during the first 4 weeks. I adjusted for baseline fungal burden estimated by cryptococcal antigen titre (using quantitative count reduced the model size by approximately half). The results are shown in Table 5.17.

Table 5.17

Cox regression model for early death (≤28 day) by MICs of Amphotericin B at 48 and 72 hours

	48 hour MIC			72 hour MIC		
Survival	**7 days**	**14 days**	**28 days**	**7 days**	**14 days**	**28 days**
Hazard Ratio	3.44	2.0	1.41	0.79	0.87	0.91
CI	0.89 – 13.3	0.8 – 4.99	0.7 – 2.8	0.29 – 2.20	0.38 – 1.98	0.45 – 1.83
P (Wald Test)	0.07	0.135	0.34	0.66	0.74	0.79
N	86	86	86	96	96	96
	Adjusted for CSF log CrAg titre					
	48 hour MIC			**72 hour MIC**		
Survival	**7 days**	**14 days**	**28 days**	**7 days**	**14 days**	**28 days**
Hazard Ratio	2.9	1.77	1.20	0.81	0.91	1.001
CI	0.76 – 11.0	0.72 – 4.36	0.61 – 2.37	0.29 – 2.3	1.03 – 1.41	0.48 – 2.09
P (Wald Test)	0.117	0.21	0.6	0.70	0.83	1.0
N	83	83	83	93	93	93

The MIC for amphotericin measured at 48 hours was associated with an increased hazard for death at 7, 14 and 21 days, and the 72 hour estimate with a reduced hazard for death at the same time points. However, no result was statistically significant, although the effect for 48 hour MIC at day 7 was close to significance. When I corrected for baseline fungal burden (CSF cryptococcal antigen titre), then the effect of MIC was reduced further.

Finally, I added the 48 and 72 hour amphotericin and fluconazole MIC data to the backward model selection developed in Chapter 3. The model examined the 6 month outcome. The coefficients associated with outcome at 6 months were unchanged, and antifungal MIC was not associated with an altered hazard for death (table 5.18).

Table 5.18

Final multivariate model for 6 month outcome after including 48 hour MICs of amphotericin and fluconazole for all patients with observations on the selected covariates (110 patients)

Variable	Hazard Ratio	Lower 95%CI	Upper 95%CI	P
Log2 Cryptococcal Antigen CSF	1.24	1.11	1.38	<0.001
GCS	0.82	0.74	0.92	<0.001
Log10 CSF white cell count	0.47	0.29	0.77	0.003

5.5 Discussion

There are no published data on antifungal susceptibility testing for *Cryptococcus* from Viet Nam. I used the YeastOne® Sensititre® system to ascertain the susceptibilities of over 400 Vietnamese clinical isolates of *Cryptococcus grubii* and *gattii* to a panel of 8 antifungal drugs. I chose the YeastOne® system because it has been shown to have good intra- and inter-operator consistency[167, 347]. The gold standard methodology for determining the MICs of antifungal drugs for yeast is the CLSI M27A broth microdilution method. The purpose of the CLSI methodology is to provide standardisation of testing systems between laboratories, and to try to ensure consistency. However, its use as a reference standard is problematic, not least because it has limited clinical utility[170, 171, 173, 174, 178, 353-355]. Moreover, while RPMI 1640 broth is recommended for the assays, in fact the use of antibiotic medium 3 (AM3) has been shown to be a better medium for determining amphotericin B susceptibilities for both *Candida* and *Cryptococcus spp*, and is preferred by some centres[113]. However, AM3 is not a standardised medium, and suffers batch to batch and manufacturer to manufacturer variation. Different nutrient concentrations and pH are known to affect the estimation of MIC [337].The YeastOne® methodology has the advantages of consistency of both plate and medium manufacture. Correlation of the YeastOne® system with the M27A protocol is considered to be good, although it varies by antifungal and species under test (table 5.19). Particularly, agreement for amphotericin B and for *Cryptococcus* species is not as good as for azole drugs and most *Candida* species[163, 167].

Table 5.19

Percent Agreement (within 2 dilutions) of 48 hour MICs measured by YeastOne® Sensititre® and CLSI M27A methods[167]

Antifungal	*Candida albicans*	Non-*albicans* *Candida spp*	*Cryptococcus neoformans*
Amphotericin B	97	92 – 99	76
Fluconazole	60	73-99	98
Flucytosine	56	73-100	96
Itraconazole	54	66-97	96
Ketoconazole	56	66-97	90

Lopez-Jodra reported 73% agreement for *C. neoformans* between 48hr CLSI amphotericin B MIC estimation and 72 hour YeastOne® estimation [334]. Davey found 89% agreement for *C. neoformans* between 72 hour CLSI amphotericin B MICs and 48 hour YeastOne® MICs [163]. Thus, while the CLSI method is regarded as the gold standard, its disappointing performance in predicting clinical outcome, other than for candidiasis with a limited number of drugs, suggest that methodologies without perfect correlation, such as the YeastOne® system, are worth evaluating. Imperfect correlation with the CLSI methodology does not necessarily imply redundancy.

5.5.1 MICs of Vietnamese isolates

MICs of drugs were estimable for 92.3 – 92.9% of isolates at 48 hours, and for 99.25 – 100% of isolates at 72 hours. I found the $\log_2$ MICs of the antifungal drugs tested to be approximately symmetrically distributed with the exceptions of caspofungin and

amphotericin B. The result for caspofungin is not surprising, since *Cryptococcus* species are inherently resistant to the echinocandin drugs.

The MICs of amphotericin B were tightly clustered, with little variation in MIC detected using the YeastOne® method. Whether this truly reflects a narrow range of susceptibilities of the *Cryptococcus* population to amphotericin, or is a result of the testing system, is not clear. It is known that with the CLSI methodology, use of different culture media can result in differences in both the absolute MICs measured and the ranges detected[113]. However, our results are consistent with other studies [111, 115, 185, 316, 333, 334, 352, 356, 357]. The susceptibility of *Cryptococcus neoformans* to amphotericin B seems to vary little with geography. Pfaller, using M27A methodology, reported MIC 90s of amphotericin of 1 mg/L for over 1800 strains from North America, Europe, Africa, the Pacific and Latin America, collected between 1990 and 2004 [352]. There was no difference by geographical region. Souza reported an MIC 90 of 1 mg/L for 110 clinical and environmental isolates of *C. neoformans* from Brazil [358]. There are fewer data from Asia. Manosuthi reported a MIC90s of amphotericin B for Thai *Cryptococcus* isolates of 1mg/L [182]. Sar reported MIC90s of 0.5mg/L for 402 Cambodian isolates, using the Etest [359]. The Etest has variable agreement with the CLSI methodology – if incubated for 72 hours, then the agreement with CLSI 72 hour MICs for amphotericin B is good (99%)[360] However, estimation of the MIC after 48 hours incubation, as used by Sar, results in a fall of the rate of agreement to 8.1% [159]. Chandenier also used the Etest, but reported 72 hour MICs of amphotericin B for 110 Cambodian isolates [112]. These were lower, the MIC90 being 0.094mg/L. Tay used the ETest (72 hour incubation) and reported MICs of amphotericin B of 0.38mg/L for 48

isolates (30 *C. neoformans* and 18 *C. gattii*) from Malaysia[361]. Archibald used the YeastOne® system to determine the MICs of 5 antifungals against 65 blood stream isolates of *C. neoformans* from the USA (n = 20), Thailand (n = 29), and Malawi (n = 16) [356]. The MIC90 of amphotericin B after 72 hours incubation was 2mg/L. There were no differences between countries.

There are no published data describing flucytosine susceptibility for *Cryptococcus* strains from Cambodia or Viet Nam. The MIC90 of flucytosine for our strains was 8mg/L at both 48 and 72 hours. The range of results was broad, varying from 0.512 mg/L to >64 mg/L. Less than half (46.1%) of our strains would be considered fully sensitive if the *Candida* breakpoints applied - for *Candida* species, an MIC of flucytosine of 8mg/L is considered to represent intermediate susceptibility to the drug. An MIC90 of flucytosine of 8 mg/L in clinical isolates is consistent with reports from Thailand, Malawi, Democratic Republic of Congo, North America and Northern Europe, but MICs of greater than 16mg/L are rarely reported [163, 334, 356]. Decreased susceptibility to flucytosine has been reported from Spain, where 46% of strains were not susceptible to 8mg/L, but in contrast, in Malaysia, the MIC of flucytosine seems markedly lower than elsewhere, reported as 0.023mg/L [361, 362].

Flucytosine is rarely used to treat cryptococcal meningitis in Viet Nam because of its cost and lack of availability. Cryptococcosis is generally not considered to be contagious – *Cryptococcus* species are not primary pathogens of humans but live freely in the environment. Thus the relatively high MICs of flucytosine seen are unlikely to be the result of drug-induced selection, but represent some inherent quality of *Cryptococcus*. However, flucytosine resistance is well described in human disease - it can develop

rapidly when used as monotherapy [363]. Despite the moderately high MICs, flucytosine combined with amphotericin B probably represents the most potent anti-cryptococcal therapy currently available (in terms of sterilising power), and there are in vitro data suggesting that even in the presence of frank resistance this combination can be potent[99, 364].

The MIC90s of fluconazole at 48 and 72 hours were 8 and 16mg/L respectively. MICs across the whole range of drug dilution were detected. Aller has reported that the clinical outcome is worse in patients receiving fluconazole maintenance therapy if the MIC of fluconazole is ≥ 16mg/L for the infecting strain ref. Over 20% of our strains had MIC's ≥ 16mg/L. However, they used dichotomised rather than continuous variables in the determination of significance in their analysis. While dichotomizing may result in a loss of power, and thus failure to show a significance, there is also a risk of falsely attributing significance if the cut points are chosen adaptively (i.e. several different cut points are tested) [251]. Pfaller reported that North American isolates had reduced fluconazole susceptibility compared with those from Africa, South America and Europe[352]. This is plausible – explainable by greater access to fluconazole for treatment/prevention of mucocutaneous candidiasis in HIV infected patients, but the spread of MICs described are very similar to our experience. Fluconazole primary prophylaxis for HIV patients in Viet Nam entered the HIV/AIDS treatment guidelines in 2006; the actual extent of prescribing is not known. However, the HIV positive isolates from 2004 onwards were all from patients entered into the RCT, for which receipt of fluconazole primary prophylaxis was one of the exclusion criteria. In a small study comparing Malawian, Thai and American isolates, Archibald found no difference in fluconazole

susceptibilities[356]. Jessup reported MICs of fluconazole for 149 Ugandan isolates, and found them to be relatively susceptible, with no isolate requiring more than 16mg/L fluconazole for growth inhibition[348]. Susceptibilities reported from Malaysia are similar to ours (but using the Etest) [361]. Much higher MICs of fluconazole have been described by Sar in Cambodia (MIC90 96mg/L, Etest), and Chandenier found MICs of fluconazole to be higher for Cambodian isolates compared with African [112, 359].I also found a small number of isolates (39) that may have had reduced susceptibility to itraconazole. In general, our isolates were more susceptible to itraconazole than has been reported in a large international series, where only 80% were inhibited by 1mg/L of drug [365]. Itraconazole has been associated with an increased risk of relapse compared with fluconazole, perhaps due to its relatively low CNS penetration [366, 367]. MICs of posaconazole and voriconazole were all low. The MIC of fluconazole was ≥64 mg/L for 5 isolates; all these isolates, which were from HIV infected patients from 2005 onwards, had probable reduced susceptibility to itraconazole (MIC 0.256mg/L, considered a dose-dependent susceptibility) and flucytosine (8 – 16mg/L, considered intermediate susceptibility). The isolates were all baseline isolates from first diagnoses of cryptococcal meningitis.

5.5.2 The effect of year and HIV status

Using linear regression I found that (with the exception of posaconazole) the geometric mean MICs of all the drugs inextricably rose over the 13 year period. The effect was most marked for ketoconazole, and smallest for amphotericin B (although the confidence intervals of the coefficients overlapped for each drug). Where the lowess

lines suggested the relationship might not be described by a straight line, I used a natural cubic spline to model the effect, and found that it was highly likely that MICs were changing over time, including for posaconazole. The actual changes in MIC over the 13 year period were relatively small (the largest, for flucytosine and ketoconazole, representing doublings of the MICs), and thus are unlikely to have any immediate clinical consequences. However, if the rise in MICs continues, then given the rates of new drug development in diseases that predominantly affect the poorer nations, these may eventually have clinical consequences. This rise in MICs over the 13 year period suggests that there is a selective pressure somewhere in the environment (clinical or extra-clinical) that *C. neoformans* var *grubii* inhabits.

I found statistically significant differences in geometric mean MICs of amphotericin, flucytosine, fluconazole and ketoconazole by the HIV serostatus of the isolate source (table 5.5). These differences were most marked between the HIV positive and unknown HIV status groups. As discussed earlier, the isolates in the unknown HIV status group are almost certainly from HIV infected patients. These 2 groups spanned different time periods. I combined these 2 groups so that I could examine for the effect of year and HIV status over the whole time period. After adjusting for year HIV infection was associated with reduced susceptibility to flucytosine (geometric mean 1.29 times higher than the geometric mean for HIV negative isolates). For the azole drugs, the effect of HIV status on MIC estimates was variable, but differences just failed to reach statistical significance. However, the effect of year remained significant for all drugs except posaconazole. I then found that the impact of time on change in MIC was different for isolates depending upon the HIV serostatus of the source for amphotericin B, flucytosine

and ketoconazole – for these drugs the rises in MIC were being seen predominantly in the HIV patients (figures 5.55 – 5.12). There were only 60 isolates from HIV negative patients, which may in part explain the differences seen. There are no published data specifically comparing MICs according to HIV serostatus of the isolate source. Again, currently the differences I have detected are unlikely to have clinical significance, but may point to the patient group in whom drug exposure drives resistance. The extent of azole drug exposure in the HIV infected patients prior to diagnosis with cryptococcal meningitis is unknown. Flucytosine exposure is extremely unlikely, since it is not generally available in Viet Nam and is expensive.

It is unclear why the MICs of the antifungals tested should rise by year. It is conventional wisdom that infection is acquired from the environment, rather than person to person. *Cryptococcus grubii* is ubiquitous; it would seem unlikely that exposure to medicinal drug is occurring anywhere in the environment sufficient to drive selection. Whether cryptococcal meningitis is a result of recrudescence of latent infection or an acute event is a matter of debate, although the incubation period can certainly be prolonged in *C. gattii* infection [73]. If the same is true for *C. neoformans* var *grubii* in HIV infected patients, then there may be the opportunity for selection of resistance, at least to azole drugs, through the use of primary prophylaxis or treatment of other fungal infections. However, the isolates in this series were from first episodes of cryptococcal meningitis, and this was frequently coincident with the diagnosis of HIV in our hospital, so a history of azole prophylaxis would be unlikely. Additionally, this would not explain the rising MICs of amphotericin B and flucytosine, not generally used in chemoprophylaxis, whose mechanism of action is different to that of the azole drugs.

Moreover, in general studies comparing drug susceptibilities of clinical and environmental isolates have failed to show significant differences [336, 358, 368]. Probably resistant (MIC >64mg/L) environmental isolates of *C. grubii* have been identified in rural and urban Spain, and the development of changing drug susceptibilities in sequential isolates from human infection has been described [158, 185, 369].

However, while exposure to medicinal antifungals in the environment is unlikely, it is possible that the evolution of drug resistance in *Cryptococcus* is driven by the use of agrochemicals. Resistance to fungicides might result in cross-resistance to medically important antifungals. Numerous non-medical azoles and imidazoles, such as ipconazole, difenconazole, hexaconazole, tricyclazole and carbendazin, have been developed to control fungal plant pathogens[370]. Of note, these compounds have long lasting stability and can remain active in soil and water over many months [370]. The use of agricultural azoles has been postulated as a source of resistance to medical azoles, and the induction of cross resistance has been demonstrated experimentally in *Colletotrichum graminicola*, a pathogen of both maize and humans [370, 371]. The MIC50 of fluconazole for *Cryptococcus neoformans* isolated from pigeon faeces from farms where non-medical azoles have been used extensively have been found to be higher than for clinical isolates from the same area in one small study [372]. Viet Nam has a vibrant agricultural industry – including rice, vegetables, tropical fruit, ground nuts, cashews, pepper, tea and coffee. It is difficult to find data concerning the use of such fungicides in Viet Nam, but other agricultural pesticides have been sought and detected in ground water both in the north and south of the country [373-375]. Viet

Nam's economic restructuring (Đổi Mới) began in 1986, allowing private ownership of farms, and economic sanctions with the United States ended in 1994. Over the past 10 – 15 years these 2 events are likely to have had a significant impact on the growth of the Vietnamese agricultural economy and access to agricultural antifungal compounds. Changes in antifungal susceptibility over time have not been detected by all investigators. Pfaller described global trends in *Cryptococcus* susceptibility over a 15 year period (1990 to 2004) in 1811 isolates [352]. No trend was found over time, although the isolates were collected from over 100 different institutions around the world, and how each institution contributed temporally to the collection is not clear, which may have led to bias, particularly if trends in susceptibility vary by geographical area. Isolates were stored as water suspensions. Brandt also reported no significant change in MIC90s over a 6 year period in 522 isolates from the USA. However, no comparison was made of geometric means, and no statistical tests were applied to the data [185]. Davey reported no significant change in MIC90s of fluconazole in 220 UK strains of *C. neoformans*, but again no formal statistical testing was used [376]. Conversely, a decrease in susceptibility over time has been reported in a short period from Cambodia, where the MIC 90 of fluconazole rose from 12 to 96 mg/L over 2 years [359]. Isolates in this case were stored at -20C in 20% glycerol. Aller reported a fall in fluconazole MIC90s in Spain between the periods 1994-1996 (23 isolates) and 1997 – 2005 (47 isolates); why this curious time distribution in this small study is chosen is not explained [377]. I used a linear model to define the effects of time and HIV on antifungal susceptibility. I reported the geometric mean as well as MIC50s and MIC90s – the geometric mean is a measure that has increased resolution compared to MIC50s

and 90s. Moreover I looked at the influence of time over the whole period rather than arbitrarily dichotomizing isolates into groups. I found that over 13 years there were statistically significant changes in this measure. These findings are consistent with the findings in another Southeast Asian country – Cambodia, and thus may be real. Changes in geometric mean may serve as early warning markers of decreasing pathogen susceptibility.

Finally, it might be that the rise in MICs is a function of storage of the isolates, and that isolates become more susceptible as they age. This has not been reported for *Cryptococcus*, or other yeasts, and would be difficult to explain biologically since the isolates are frozen and therefore presumably inert. This question could only be answered prospectively.

5.5.3 Effect of Species

All but 16 isolates were identified as *Cryptococcus grubii* molecular group VN1, using *URA5* PCR-RFLP as previously described. The remaining 16 isolates were *Cryptococcus gattii* (14 *URA5* PCR-RFLP molecular group VG1 and 2 molecular group VG2). The geometric mean MICs of amphotericin B and flucytosine were lower for the *C. gattii* strains compared with the *C. grubii*. I detected no significant differences for the azole drugs. Again, this is unlikely to be the result of drug pressure. The differences between the antifungal susceptibilities of *C. gattii* and *C. neoformans* are not clear. Most comparative series, like mine, have small numbers of *C. gattii*. Initially, no differences were found [378]. *C. gattii* from both clinical and environmental sources has been reported to be associated with reduced susceptibility (higher geometric mean MICs) to

antifungal drugs, including amphotericin, flucytosine and fluconazole [68, 124, 379, 380]. Our experience, although limited by small numbers, is consistent with reports from Spain and Malaysia, and Thailand [361, 381, 382]. It may be that susceptibility of *C. gattii* varies by geographical area; however, reports have been contradictory even from within small well-defined geographical areas [379, 381]. Treatment guidelines for patients with *C. gattii* are influenced by the larger body of evidence relating to *C. grubii / neoformans*. Given this, the lack of strong evidence of clinically meaningful reduced susceptibilities of *C. gattii* is reassuring.

In chapter 4 using AFLP I identified a clade of *Cryptococcus grubii* which may be more able to cause disease in HIV uninfected patients, suggesting relatively increased virulence compared with the whole *C. grubii* population. It is reassuring that I found no difference in the antifungal susceptibility of these strains compared with the general population.

5.5.4 Prediction of MICs.

The rate of difference between 48 and 72 hour MIC for all the drugs tested was high. Using linear regression I was able to predict the 72 hour MICs of all the antifungal drugs with high accuracy (to within 1 fold dilution) – 99.5% for amphotericin B, 98.6% for flucytosine and 97.8% for fluconazole. However, around 8% of strains have no discernible growth at this stage, but can still have what might be considered reduced susceptibility at 72 hours. The 72 hour results for these 32 isolates are shown in table 5.20. Isolates with potentially reduced susceptibility are marked in bold. Whether it is worth trying to predict 72 hour MICs from 48 hour MICs would depend upon whether

the 72 hour MIC has more clinical value than the 48, and whether a clinical intervention 24 hours sooner (such as dose modification or change of drug combination) has any clinical benefit.

I used linear regression to try to predict the MICs of itraconazole, posaconazole and voriconazole from the fluconazole MIC. Since they are the same class of drug, with the same mechanism of action (inhibition of sterol synthesis), then it is reasonable to assume there may be a predictable relationship between drug susceptibilities. If it is possible to predict the MIC of one drug from another, then there are potential cost savings. Prediction accuracy to within a 1 fold dilution was reasonable for voriconazole and posaconazole (94.5 and 96% respectively), but was less good for itraconazole (90.2%). The error rate for a difference of 3 or more dilutions for itraconazole predictions was 1.5%. It may be reasonable to predict other azole drug MICs for *Cryptococcus* based on the YeastOne® fluconazole result, at least if the fluconazole MIC is of a value where a prediction error is unlikely to define erroneous sensitivity to the other azoles. This has been suggested as a strategy to identify *Candida spp* susceptible to posaconazole, where fluconazole and voriconazole MICs (using the M27A methodology) predict the MIC with an accuracy similar to that which I have described [383].

Table 5.20.72 hour MICs, ug/mL, of 4 antifungal drugs for 32 isolates where there was insufficient growth at 48 hours for estimation.

Isolate*	Amphotericin	Flucytosine	Fluconazole	Itraconazole
1	0.512	8	16	0.256
2	0.512	4	8	0.128
3	0.512	4	8	0.128
4	0.512	8	2	0.004
5	0.512	16	16	0.016
6	0.512	8	4	0.032
7	0.512	4	4	0.064
8	1.024	4	16	0.128
9	0.256	8	1	0.004
10	1.024	2	16	0.128
11	0.512	8	16	0.064
12	0.064	8	4	0.032
13	0.512	8	32	0.512
14	1.024	16	32	0.256
15	0.512	4	0.125	0.004
16	0.512	8	4	0.064
17	0.512	2	4	0.032
18	0.256	2	2	0.032
19	0.512	8	4	0.064
20	0.512	0.96	16	0.032
21	0.512	8	16	0.256
22	1.024	2	2	0.128
23	0.256	4	8	0.064
24	0.512	0.96	4	0.032
25	0.512	2	1	0.004
26	0.512	4	4	0.004
27	0.512	4	8	0.016
28	0.256	2	16	0.064
29	0.256	4	4	0.128
30	0.512	2	4	0.016
31	0.128			0.032
32				0.064

5.5.5 Effect on CSF sterilisation

We examined the effect of amphotericin B MIC on the rate of decline of yeast colony forming units in CSF over the first 28 days of therapy. Rates of decline in fungal quantitative count in CSF have been pioneered by Harrison and colleagues as a potential investigative tool for comparison of antifungal therapies in cryptococcal meningitis [99, 138, 186, 205]. Sampling CSF on 4 set occasions over the first 2 weeks of treatment (days 1, 3 , 7 and 14), they have demonstrated differences in the rates of decline of yeast colony forming units according to the treatment given[99]. They report declines in viable yeast counts in CSF of 0.31 $\log_{10}$ - 0.45 $\log_{10}$ CFUs/day over the first 14 days of therapy in patients receiving amphotericin B 0.7mg/kg/day monotherapy and 0.56 $\log_{10}$ for patients receiving 1 mg/kg/day [99, 137]. This compares with the fall of 0.32 $\log_{10}$ CFUs/day seen in the patients in this study, who were receiving 1mg/kg/day. The Vietnamese patients had significantly higher baseline yeast burdens than the patients reported by Harrison's colleagues, which has been associated with lower rates of clearance [99, 137]. More recently, Bicanic analysed a combined dataset of 262 of their patients drawn from randomized controlled trials, non-randomised trials and case series, to investigate the influence of the rate of yeast clearance on outcome [138]. In a multivariate model including altered mental status at baseline, baseline fungal count and the rate of clearance of infection, all 3 factors were independently associated with mortality. Each 0.1log10-unit decrease in the rate of clearance in fungal load was associated with an increase in the hazard ratio for death of 1.34 (95% CI 1.06 – 1.68, p = 0.01) at 2 weeks and 1.18 (95% CI 1.04 – 1.33, p = 0.008) at 10 weeks. However, when the serial colony-forming unit counts were fitted as a time dependent variable, after

adjusting for baseline fungal load and mental status, an increase in the hazard ratio for death was seen only at 2 weeks (HR 1.82, 95%CI 1.12 – 2.96; p=0.007 at 2 weeks versus HR 1.13, 95%CI 0.65 – 1.32; p=0.14 at 10 weeks, for each unit increase in the last log10 colony forming unit count) [138]. The combination of patients from outside clinical trials may have introduced bias onto this analysis. In particular, data were used from an observational study of 54 South African patients where the Glasgow Coma Scores determined treatment - all those with scores ≥ 10 received amphotericin B, those with scores less than 10 received fluconazole 400mg/day monotherapy[205]. This treatment allocation reflected a conception that patients with coma scores less than 10 had very poor prognoses, and therefore did not receive amphotericin which was in short supply. It is conceivable that this bias may have lead to difference in other aspects of care with adverse consequences. Sixty patients were recruited from a trial of differing doses of fluconazole in Uganda[384]. The treatment allocation was not randomized. There were some differences between the patients in my study and those analysed by Bicanic. Unlike Harrison's group, I sampled patients over the first 4 weeks of therapy, and looked at 6 month outcome. Notably, the median CSF yeast count at baseline in our patients is 10 fold higher (log10 6.02 vs. log10 5.4) CFU/ml, and the median CD4 counts in our patients were lower, (median 14.5 versus 26 cells/uL). The higher fungal burden at baseline in our patients suggests our patients may have been generally more severely unwell than those in Harrison's combined analysis.

For our data, an extended model best explained the rates of decline. We did not show any effect of the rate of decline of fungal cell count on the hazard for death at 6 months. Modelling the data at an earlier time point (such as 2 or 4 weeks) is methodologically

difficult, since my study size is small and there are insufficient events (deaths) by these time-points.

We failed to show an association between amphotericin B MICs measured at either 48 or 72 hours and the rate of decline in yeast count. However, the range of amphotericin B MICs, as described earlier, was narrow. It is feasible that the yeast count methodology is insufficiently sensitive to detect differences in decline due to small changes in MIC. As would be expected, we found no association between fluconazole susceptibility and the rate of decline in fungal count - no patients were receiving fluconazole during the first 4 weeks of treatment. In the future we will perform these analyses on a larger dataset.

5.5.6 Clinical Outcome

The clinical impact of yeast sensitivity testing has been disappointing in cryptococcal meningitis. Aller reported a small series (25 patients) in whom an MIC of ≥16mg/L was associated with therapeutic failure[183]. Therapeutic failure was defined as positive cultures at 10 weeks or at the time of death. Only 5 patients had therapeutic failure, and the small study size precluded robust statistical analysis, but those patients who died had higher cryptococcal antigen titres, which I have found to be the most powerful prognostic indicator, and were more likely to have had previous cryptococcal meningitis. In addition, as mentioned before, the MICs were dichotomised for analysis.

Bicanic reported 21 culture positive cryptococcal meningitis relapse patients who had previously been treated with fluconazole monotherapy. 14 of 21 isolates had probable fluconazole resistance, with MICs of fluconazole of ≥ 64mg/L [195]. All patients were retreated with prolonged courses of amphotericin. Seven of the patients with resistant

isolates died within 6 months of follow-up. The time of death in relation to the administration of amphotericin B is not described (i.e. it is not clear whether the patients died during retreatment with amphotericin). No isolates were available from the first episode of illness, so the antifungal susceptibilities at first presentation are not known. Thus it is not known whether these relapses would have been predicted by MIC testing at first presentation.

Witt reported 76 patients of whom 19 had therapeutic failure [349]. In a multivariate analysis, of 3 variables (presence of positive blood culture, flucytosine therapy and fluconazole MIC), the MIC of fluconazole was the least significantly associated with therapeutic failure (p=0.012, 0.011 and 0.039 respectively). When the serum cryptococcal antigen titre was used as a covariate instead of blood culture result, the MIC of fluconazole lost statistical significance (p=0.003, 0.005 and 0.054 respectively). Fluconazole MICs were measured using the M27A methodology. Conversely, numerous large studies have failed to show a relationship between antifungal MICs and outcome in cryptococcal meningitis [114, 161, 182, 348]. This contrasts with severe candidiasis, where the majority of studies have shown the MICs of antifungals to have some prognostic value [171, 173, 175, 177-180, 385]. I examined the association of antifungal MIC in a subset of patients (124) for whom we had extensive clinical and outcome data (Chapter 3). I had identified fungal load, estimated by either CSF viable yeast count or CSF cryptococcal antigen titre, as the factor most highly associated with an increased hazard of death. All the patients were treated with amphotericin B followed by fluconazole. Neither 48 hour nor 72 hour MICs of amphotericin B or fluconazole had any impact on the hazard for death at 10 weeks. I also analysed the effect of

amphotericin B MIC on the hazard for death at the 7, 14 and 28 day time points, assuming that early deaths may be more likely to be due to lack of effective yeast killing. I failed to show a statistically significant association, although the coefficient for the hazard declined plausibly over the 4 week period, from 3.44 ($p = 0.07$) at 7 weeks to 1.141 at 28 days for the 48 hour estimation. Interestingly, the 72 hour MIC estimation had no impact on the hazard for death. After correcting for fungal burden (using cryptococcal antigen titre in CSF), the size of the effect of the amphotericin B MIC (if any) was further reduced. The early predictive effect of 48 hour amphotericin B MIC needs to be reassessed in a larger dataset. It might be that there is a subset of patients who clear yeast slowly in whom I have missed an effect.

In summary, using the YeastOne® method I could detect changes in MIC over time, by HIV serostatus and by species for different antifungals, but I was unable to relate MICs to the rate of CSF sterilization or the clinical outcome, although the results for 48 hour amphotericin B suggest that this should be evaluated in a larger dataset.

Chapter 6
Discussion

The aim of this thesis was to address the following questions:

1. Is the clinical phenotype of cryptococcal meningitis influenced by HIV infection?
2. What are the prognostic factors in cryptococcal meningitis?
3. What is the molecular epidemiology of human isolates of *Cryptococcus neoformans* in Viet Nam, and is this influenced by HIV serostatus?
4. Has the antifungal susceptibility of *C. neoformans* changed over time, do the susceptibility profiles of *C. neoformans* differ by infecting variety and immune status of the host, and what is the clinical utility of antifungal sensitivity testing in cryptococcal meningitis.

The extent to which these questions have been answered will now be discussed.

6.1 Clinical Phenotype and Prognostic Factors

I first compared the literature on the clinical phenotypes of patients by HIV infection status. From randomised controlled trials and descriptive studies I found clinical data describing a total of 2204 patients. Concatenating the data from these individual studies suggested that there were indeed some differences in clinical presentation according to HIV serostatus. Neck stiffness, confusion, focal neurological signs, visual impairment

and nausea and vomiting were all more common features of disease in HIV uninfected patients. However, within each group (HIV infected or uninfected) I found that the patient populations were highly heterogeneous. In particular, the patients in van der Horst's study, the largest ever randomised controlled trial in cryptococcal meningitis, and the study which forms the basis for the published treatment guidelines, had low rates of headache and fever compared with other study groups, suggesting that this patient population was significantly different to most other reported HIV infected patient populations with cryptococcal meningitis. As has been mentioned earlier, these patients seem to have had less severe disease than most patients with cryptococcal meningitis, probably as a direct result of the inclusion and exclusion criteria for the study. This is mirrored in the relatively low death rate of around 10%. Other reports of cryptococcal meningitis from the USA have reported higher death rates more in keeping with experience elsewhere [100, 142].

Within the HIV uninfected patients, again, marked heterogeneity was seen between the study populations. This is perhaps easier to explain - most reports of cryptococcal meningitis in HIV uninfected patients are from populations with a diverse range of immunosuppressing conditions including haematological malignancy, solid organ transplantation, connective tissue disorders and treatment with a wide range of immunosuppressive drugs. In addition some studies include patients with *C. gattii* infection.

The heterogeneity in both the HIV positive and HIV negative study populations means that it is methodologically problematic to concatenate their data, and the differences that appear to exist between clinical phenotypes cannot be considered reliable. In addition it can be argued that this heterogeneity means that it is difficult to extrapolate results of randomised controlled trials from one patient population to another, and underlines the importance of minimising exclusion criteria in order to ensure that the results of clinical trials remain applicable to the general population. In the recent Cochrane review of treatment of cryptococcal meningitis, there was no attempt made to define inter-study population heterogeneity [386]. One conclusion drawn by the authors is that a difference in mortality between North American patients and those elsewhere in the world is due to different management of raised intracranial pressure [107, 386]. However, studies from less well resourced settings, such as Thailand, Papua New Guinea, and my experience presented here have found that raised intracranial pressure at baseline is not independently associated with outcome, suggesting that even in resource poor areas this common complication can be managed appropriately [62]. Different severities of disease at presentation may better explain differences in mortality rates between studies than management of raised intra cranial pressure, although access to treatment other than azole drugs is likely to be the most important factor.

At the Hospital for Tropical Diseases, cryptococcal meningitis in both HIV infected and HIV uninfected patients is seen. I investigated the impact of HIV on the clinical presentation of cryptococcal meningitis by comparing baseline historical, clinical and investigation findings between 2 cohorts of patients, which differed by HIV infection

status. With the exception of disease due to *C. gattii*, the clinical phenotype of cryptococcal meningitis in HIV uninfected patients is frequently complicated by underlying disease with its own significant morbidities and mortalities, and this complicates any determination of the effect of HIV on clinical presentation. At HTD, cryptococcal meningitis in HIV uninfected patients is of particular interest on two counts; first, most patients (81%) have no underlying disease. Of eleven patients with co-morbidities, the most frequent predisposing factor was corticosteroid use in 7 patients (one with SLE, two with nephrotic syndrome, 2 with unclassified chronic arthritides and two with Evan's syndrome). Of the remaining patients, 2 had cirrhosis, one had cirrhosis and renal impairment, and 1 had diabetes mellitus. Whether diabetes mellitus predisposes to cryptococcal disease is not clear [4, 387]. The diseases seen in this series arguably have less impact on immune function compared to haematological malignancy and its therapy, and the drugs used in organ recipients. While cirrhosis, SLE and diabetes can all cause CNS complications, their frequency in the cohort was low. Secondly, as I found in Chapter 4, most HIV uninfected patients (75%) have disease due to infection with *C. neoformans* var *grubii*, the commonest cause of disease in HIV infected patients and worldwide. To define the impact of HIV on cryptococcal meningitis ideally requires a comparator group of HIV uninfected patients, from a similar patient population (i.e. the same geographical area and ethnic group), without co-morbidities, and infected with the same species of *C. neoformans*. Thus, while my comparison was not perfect, the HIV negative cohort was closer to fulfilling these requirements than many other studies. I could have excluded patients with underlying disease, or *C. gattii* infection, but the power of the comparison would have been

reduced. In the future, as more HIV uninfected patients are diagnosed, it may be possible to repeat this analysis excluding the *C. gattii* cases and those with pre-existing co-morbidities.

I found that there were differences in the demographics between the HIV infected and uninfected patients, reflecting the demographics of the AIDS epidemic in Viet Nam, which is predominantly a disease of young male injection drug users. Duration of symptoms was shorter in HIV infected patients, headache was more frequent, and papilloedema less common, but otherwise there were few differences in the disease presentation by HIV serostatus. Laboratory investigations were suggestive of differences in the degree of inflammation within the CNS, and fungal burden was higher in the HIV infected patients.

The mortality was significantly higher in HIV infected patients. However, the shapes of the Kaplan-Meier curves were similar for both groups of patients, with most deaths occurring within the first 4 weeks of treatment. This suggests that in the HIV patients most deaths are accounted for by *C. neoformans*, rather than other opportunistic infections. It is not possible to say whether the exact causes of death (for example, failure of sterilisation, persistence of raised intracranial pressure) are the same between the groups, and this will be an area for future study.

A plethora of prognostic indicators have been defined for cryptococcal meningitis, which is probably a reflection of the research methodologies used. Prognostic factors have been derived from prospective descriptive studies, randomised controlled trials and

retrospective case series. Without predefined hypotheses concerning the prognostic factors, and with testing of multiple variables, there is always the risk of defining false associations. My work is the first systematic assessment of previously defined prognostic factors in cryptococcal meningitis. Of over 40 identified factors only six retained prognostic significance on univariate analysis. Interestingly, most factors found to be predictive of outcome at 10 weeks were also predictive of outcome at 6 months. More variables retained prognostic significance for the HIV uninfected cohort.

The multivariate analysis identified 5 factors associated with outcome at 6 months. Those most highly associated with outcome were the cryptococcal burden (indicated by the antigen titre), the Glasgow Coma Score, and the CSF white cell count, all of which are biologically plausible, and relatively simple to measure. Future work will include determining whether these factors can be combined into a score in order to be able to assign patients to prognostic groups at presentation, similar to the Medical Research Council (UK)'s grading system in tuberculous meningitis [129].

6.2 Molecular Epidemiology

I was interested in how *Cryptococcus* strains from HIV uninfected patients related to those from HIV patients. I found that almost all cryptococcal meningitis in Viet Nam is due to infection with one *URA5I* RFLP molecular subtype of *C. neoformans* var *grubii*. Despite our tropical location, and the widespread presence of eucalyptus trees, infection with *C. gattii* is extremely rare.

I used AFLP to define the relationships of the strains. AFLP has the advantages of being a whole genome based typing system with high resolution, and does not require any prior knowledge of genome sequence. A disadvantage of AFLP is that it there is an inbuilt variability rate due to experimental factors that cannot be completely eliminated, and unlike a sequence based method, cannot necessarily be detected. The consistency I achieved in re-typing single isolates, of between 95 and 98%, compares favourably with the literature [246, 388, 389].

I used 2 primer sets, and each primer set identified a clade of *C. neoformans* var *grubii* with increased propensity to infect HIV uninfected patients. The structures of the trees were well supported with high cophenetic correlation values at the nodes defining clades. However, when I assigned band classes to the data, I was unable to demonstrate reliable nodes defining clades and sub-clusters using a bootstrap analysis. This is most likely because the strains are very closely related, and the differences between strains are defined by a large number of infrequent bands. Using a band-based analysis resulted in significant loss of information, and reduced resolution of the AFLP typing. This is not particularly concerning, because the defining of band classes is essentially arbitrary and open to operator bias.

Prior to this work, the hypothesis that disease in HIV uninfected patients might be due to infection with a sub-type of *Cryptococcus neoformans* of increased virulence was met with some scepticism, the dogma being that disease risk is determined by immune status [4, 64]. It is obvious, thanks to the HIV epidemic, that immune status is extremely important in determining the risk of disease due to *Cryptococcus neoformans* in most cases [1]. However, my work suggests that there is a clade of *Cryptococcus neoformans*

var *grubii* that has increased propensity to cause disease in immunocompetent patients. This clade cannot be dominant in the environment, since it is less common in HIV infected patients than other *C. neoformans* var *grubii* strains. The situation is analogous to that seen with *Cryptococcus gattii*. It is clear that this pathogen occurs in areas where there is a high incidence of HIV infection, yet overt disease is rare [70, 85, 87, 259]. It seems more plausible that the frequency of disease in the HIV infected is low because a disease producing exposure to the pathogen is rare, rather than disease only being able to occur when there is also some other undefined immune deficit present. Whether the clade I have identified occupies a particular environmental niche is not clear, but it seems likely that its increased pathogenicity is a by-product of some adaptation to its environment. Identifying its ecological niche would be an interesting project and may form part of future work.

I have not been able to answer the question whether infection with the newly identified clade is associated with an increased risk of death or severe disease. However, I expect to be able to answer this question in the future, when a trial I am running in 300 patients with cryptococcal meningitis is completed (ISRCTN95123928, http://www.controlled-trials.com/ISRCTN95123928/farrar). It may be that this clade is better able to establish infection in the first instance and, once established, disease has a similar phenotype to disease in *C. gattii* infection or in HIV patients. An increased ability to establish infection might simply be due to increased production of basidiospores (and thus a higher infective dose), or adapted basidiospores that are better able to withstand host immunity or invade mucosal epithelium. Alternatively, the clade may be rare in the environment, but undergo mating behaviour more frequently. Understanding the

prevalence of the clade in the environment in relation to other clades would help answer this question.

The clade may also be more pathogenic in the sense that it can cause more severe disease. My preliminary data do not support this – fungal burdens in patients were no different by clade, although complicating the data there were more underlying diseases in patients affected with clade 2 isolates. Interestingly, in the HIV negative patients for whom clinical clade data were available, clade 2 isolates only occurred in those with underlying disease. A more sophisticated analysis, when more data are available, is needed.

Sequence based typing methods, such as multi locus sequence typing (MLST), have the advantage of being rather more verifiable than band based methods, where homoplasy reduces discrimination. In addition, the evolutionary relationship between strains can be better defined. I have begun 7 locus MLST typing of the isolates to confirm the findings of the AFLP typing [305]. If this is successful, I will develop a PCR to enable rapid identification of strains from clade 1. This should also help determine whether clade 2 is monophyletic, as suggested by the GT primer derived tree, or whether there are a number of other clades circulating.

The identification of the clade offers interesting possibilities to improve the understanding of the pathogenicity of *C. neoformans*. The first step would be whole genome sequencing of representative strains from each clade to determine the degree of diversity within the clades. The arrival of the latest generation sequencer platforms

would enable this to be done at reasonable cost. A platform such as the 454 (454 Life Sciences, Branford, Connecticut, USA) could be used to assemble the genomes from 1 or 2 representative isolates from each clade, and then a more economic platform, such as SOLiD (Life Technologies, Carlsbad, California, USA) , could be used to sequence the remaining strains and determine the intra-clade diversity. The ultimate purpose of sequencing would be to better understand how *Cryptococcus* causes disease. Yeasts are complicated pathogens which alter their gene expression depending upon their environment. For example, *Cryptococcus neoformans* rarely produces significant capsule if grown on Sabouraud's agar. Thus in vitro experiments are probably a poor model of cryptococcal pathogenicity, and may be misleading. Similarly, the mouse model is one of disseminated rather than central nervous system infection, and again is not necessarily extrapolable to human disease [390-392]. However, the mouse model does seem to be able to reliably detect changes in virulence of mutant knockouts of previously identified virulence factors [393]. Because in cryptococcal meningitis *Cryptococcus* is found in significant numbers in cerebrospinal fluid, using next generation sequencing technology it will be possible to determine quantitative gene expression in human CSF. This offers the chance to confirm or refute the results of in vitro and animal work on virulence, to identify new virulence factors related to particular clinical phenotypes, and to identify new drug targets. Gene expression, through RNA sequencing, could be compared according to infecting species, clade and HIV serostatus. I plan to take this research forward through a fellowship application.

6.3 Antifungal Susceptibility

The value of antifungal sensitivity testing in cryptococcal disease is unclear. Potential uses of susceptibility testing include surveillance - early warning of falling susceptibilities in the population prior to the development of frank resistance - and as an aid to treatment selection (both drug and dose) in individual patients. The value of any susceptibility testing method ultimately depends upon how accurately it models pathogen growth within the human host. So far, antifungal susceptibility testing of *Cryptococcus neoformans* has had little tangible benefit for patients.

In the first instance, I wanted to determine whether the susceptibility of *Cryptococcus neoformans* was changing with time, and whether there were differences in susceptibility by species and by the patient population from which the isolate came. Due to the spread of the HIV epidemic, and increased use of azoles for non-cryptococcal disease in these patients, it was feasible that there would be differences in susceptibility to these drugs over time, and by patient group. Additionally, I wanted to determine whether susceptibilities measured by the YeastOne method correlated with outcome.

American studies have found no changes in the MICs of *Cryptococcus neoformans* over the years [352]. I found that, as measured by the geometric mean MICs of a panel of antifungal drugs, *Cryptococcus neoformans* var *grubii* is becoming less susceptible to key antifungals of all classes year on year. Currently, this is unlikely to have clinical significance, since the MIC90s of the drugs are still comfortably within the therapeutic range. However, this phenomenon suggests that there is a selection pressure occurring somewhere within the environment of *C. neoformans*. This pressure, as discussed in

Chapter 5, could be present either within patients, particularly if cryptococcal meningitis truly represents recrudescence of latent infection, or somewhere within the natural environment that *C. neoformans* inhabits.

Cryptococcal meningitis is coincident with the diagnosis of HIV infection for most patients in our hospital. Thus it seems unlikely that exposure to azole drugs is occurring within the patient. It is also unlikely that decreased susceptibility is due to infection of a patient with a strain that has been previously exposed to drug within anther patient – cryptococcal meningitis is not thought to be contagious. It is not impossible that drug exposure is occurring in patients on long term suppressive therapy within sanctuary sites that then have access to the environment. For example, the prostate is a recognised site of asymptomatic infection with *C. neoformans* [147]. Viable yeast could presumably be shed in the urine and re-enter the environment. However, *Cryptococcus neoformans* is ubiquitous. It seems more likely that there is exposure to azole-type drugs occurring somewhere in its primary environment. In Viet Nam, this is plausible. As we have seen, numerous azoles are used in agriculture to control fungal pests. Viet Nam has a large and vibrant agricultural sector. The use of chemicals in farming in Southeast Asia is believed to be widespread – Taiwan, Korea and Japan have recently banned the importation of Chinese fruit and vegetables because of such concerns (http://www.associatedcontent.com/article/625003/), and there is sufficient concern within Viet Nam such that there is now a fledgling indigenous organic movement (although whether this has health benefits is a moot point). Regulation and enforcement of the use of agricultural chemicals is difficult in Viet Nam, because most tropical fruit, nut and rice production is done by small farms and smallholdings. There are already

experimental data that suggest that exposure of shared plant/human pathogens to agricultural azoles can result in reduced susceptibility to medicinal azoles [371]. Furthermore, the use of azoles on farms has been associated with the recovery of *Cryptococcus neoformans* from bird guano with reduced susceptibility to medical azoles in Brazil [372]. It would be interesting to see if this work could be repeated in Viet Nam.

Why the susceptibility of *C. neoformans* var *grubii* to amphotericin B and flucytosine is also decreasing might seem more difficult to explain. Amphotericin B binds to sterols in the fungal cell membrane. Production of these sterols is inhibited by azole drugs. The mechanisms of action of the drugs are thus linked, and therefore it is feasible that some adaptation that confers azole resistance may also impact upon the efficacy of amphotericin, whether that is reduced binding sites for the drug or reduced affinity. In fact, altered sterol metabolism has been shown to confer cross resistance between fluconazole and amphotericin B in both *Candida albicans* and *Cryptococcus neoformans* isolates from humans [394, 395].

It is less obvious, but also feasible, that azole resistance, and therefore azole exposure, can be linked to flucytosine resistance. It has been shown in clinical isolates of *Candida lusitaniae* that mutations in the *FCY1* and *FCY2* genes that encode purine –cytosine permeases result in cross-resistance to flucytosine and fluconazole [396-398]. This has not yet been described in *Cryptococcus neoformans*, but warrants investigation.

I did not demonstrate changes in *C. gattii* MICs over time. The number of *C. gattii* isolates was small, and thus I had very little power to show a change if present, but it is

also likely to be rare in the environment and thus have less instances of exposure to drug pressure. *C. gattii* appeared to be more susceptible to amphotericin B and flucytosine than *C. neoformans* var *grubii*. This is similar to reports from Malaysia [361].

I could find no differences in drug susceptibility by *C. neoformans* var *grubii* clade other than for posaconazole.

These analyses made a large number of comparisons. The changes in MIC over time, while not yet clinically significant, are convincing in that there are consistent trends for several drugs. However, given the number of comparisons made, some of the differences detected may be the result of chance. The finding of decreased susceptibility of clade 1 isolates to posaconazole seems likely to be such a spurious finding, given that there are no differences between susceptibilities to other drugs, and the p value is only 0.04.

Only flucytosine susceptibility seemed to be associated with the HIV serostatus of the isolate – flucytosine MICs were higher in HIV associated cases. There was no such association for amphotericin or any azole drug.

Despite there being many methodologies now available which can reproducibly describe the MIC of a drug against a particular cryptococcal isolate, measuring antifungal susceptibility has not been shown to correlate consistently with clinical outcome. The exception is azole susceptibility in relapse isolates. The prognosis of relapsed patients is poor, but it has proved difficult to define this group of patients at baseline with sensitivity testing. Given the fact that azole resistance is potentially linked to cross-resistance to both amphotericin and flucytosine, the ability to predict azole failure may have important utility in defining a group of patients who should receive a longer initial

duration of therapy with amphotericin and flucytosine, particularly if the period of treatment where cross resistance is selected is the azole phase of treatment. The probability of developing/selecting resistance depends upon the size of the population. Initial therapy that has a greater effect on reducing the biomass of pathogen in the CSF and brain may be key in reducing the risk of treatment failure.

I used two measures to assess the usefulness of MICs of antifungals as determined by YeastOne in determining outcome – the rate of clearance of fungal load, and survival. I found that the decay of yeast CFUs within the CSF over 28 days was best defined by a 2 phase model. I was unable to detect a difference in the rate of decline of yeast CFUs in CSF according to amphotericin MIC. I also tested the effect of fluconazole MIC on the rate of decline and, perhaps as expected since no patients were receiving this drug, no effect was seen. The model had over 200 data points from 63 patients. If the effect of amphotericin MIC is small, then I may have missed an effect. Future work will include measuring the effect of fluconazole and flucytosine susceptibilities on fungal decay in patients receiving those treatments.

Finally, I looked at survival in relation to drug susceptibility. I could find no alteration in the hazard for death at 70 days depending upon the isolate susceptibility to amphotericin, fluconazole or flucytosine. However, when I examined the effect of 48 hour amphotericin MIC estimation at days 7, 14 and 28, it appeared that there might be a relationship between amphotericin susceptibility and the hazard for death. The results did not reach conventional levels of significance, but the work needs to be repeated on a larger dataset, which is planned. Of note, most protocols recommend estimating the

amphotericin MIC for *Cryptococcus* isolates after 72 hours incubation. The MIC measured at this time point had no relationship with the hazard for death.

6.4 Concluding Comments

The burden of cryptococcal disease is huge, and concentrated in poorly resourced countries. While the vast majority of cases occur in HIV infected patients, disease is not limited to this group. The development of increasingly effective immunosuppressive treatments to manage chronic inflammatory disease and organ transplantation, and the ubiquity of *C. neoformans* means that disease in the HIV uninfected will always be present. It is not only the immunosuppressed who are at risk - the British Columbia outbreak has demonstrated that *C. gattii* can establish itself in new environmental niches and cause significant disease.

Despite the large numbers of cryptococcal meningitis patients, and the failure of any major advance in treatment since the advent of fluconazole, there is little concerted effort to run randomized controlled trials powered to clinical endpoints. The last trial powered to clinical endpoints was that of van der Horst, published in 1997. I have shown in this thesis that the populations of patients with cryptococcal meningitis are highly heterogeneous, and therefore we cannot necessarily extrapolate results from one patient group to another. There is a need to continue clinical research powered to meaningful endpoints in both HIV infected and uninfected patients.

The most exciting result of this PhD is that I have demonstrated the presence of a sub-clade of *C. neoformans* var *grubii* that has the ability to cause serious disease in apparently immunocompetent patients. While its ecological niche is not known, it is likely to be associated with pigeons, and there is potential for dispersal of the pathogen. It offers a great opportunity to better understand the pathogenesis of cryptococcal meningitis, which I hope to take forward in the future.

References

1. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM: **Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS.** *Aids* 2009, **23**(4):525-530.
2. Sanfelice F: **Contributo alla morfologia e biologia dei blastomiceti che si svilupano nei succhi di alcuni frutti.** *Ann Igien* 1894, **4**:463 - 495.
3. Sanfelice F: **Sull'azione patogena dei blastomiceti.** *Ann Inst Igien Univ Roma* 1895, **5**:239 - 262.
4. Casadevall A, Perfect JR: ***Cryptococcus neoformans***, 1 edn. Washington: American Society for Microbiology Press; 1998.
5. Vuillemin P: **Les blastomycètes pathogènes.** *Rev Gen Sci Pures Appl* 1901, **12**:732 - 751.
6. Bovers M, Hagen F, Boekhout T: **Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex.** *Rev Iberoam Micol* 2008, **25**(1):S4-12.
7. Todd RL, Hermann WW: **The life cycle ofthe organism causing yeast meningitis.** *J Bacteriol* 1936, **32**:89 - 103.
8. Mooke WH, Moore M: **Cutaneous torulosis.** *Arch Derm Syph* 1936, **33**:951 - 962.
9. Benham RW: **Cryptococcosis and blastomycosis.** *Ann NY Acad Sci* 1950, **50**:1299 - 1314.

10. Mitchell TG, Perfect JR: **Cryptococcosis in the era of AIDS--100 years after the discovery of *Cryptococcus neoformans***. *Clin Microbiol Rev* 1995, **8**(4):515-548.

11. Freeman W: ***Torula* infection of the central nervous system.** *Journal of Psychology and Neurology* 1931, **43**:236 - 345.

12. Furman-Kuklinska K, Naumnik B, Mysliwiec M: **Fungaemia due to *Cryptococcus laurentii* as a complication of immunosuppressive therapy--a case report**. *Adv Med Sci* 2009, **54**(1):116-119.

13. Khawcharoenporn T, Apisarnthanarak A, Kiratisin P, Mundy LM, Bailey TC: **Evaluation of *Cryptococcus laurentii* meningitis in a patient with HIV infection: a case report and review of the literature**. *Hawaii Med J* 2006, **65**(9):260-263.

14. Kordossis T, Avlami A, Velegraki A, Stefanou I, Georgakopoulos G, Papalambrou C, Legakis NJ: **First report of *Cryptococcus laurentii* meningitis and a fatal case of *Cryptococcus albidus* cryptococcaemia in AIDS patients**. *Med Mycol* 1998, **36**(5):335-339.

15. Lee YA, Kim HJ, Lee TW, Kim MJ, Lee MH, Lee JH, Ihm CG: **First report of *Cryptococcus albidus*--induced disseminated cryptococcosis in a renal transplant recipient**. *Korean J Intern Med* 2004, **19**(1):53-57.

16. Evans EE, Kessel JF: **The antigenic composition of *Cryptococcus neoformans*. II. Serologic studies with the capsular polysaccharide**. *J Immunol* 1951, **67**(2):109-114.

17. Evans EE: **An immunologic comparison of 12 strains of *Cryptococcus neoformans* (*Torula histolytica*).** *Proc Soc Exp Biol Med* 1949, **71**(4):644-646.

18. Evans EE: **The antigenic composition of *Cryptococcus neoformans*. I. A serologic classification by means of the capsular and agglutination reactions.** *J Immunol* 1950, **64**(5):423-430.

19. Wilson DE, Bennett JE, Bailey JW: **Serologic grouping of *Cryptococcus neoformans*.** *Proc Soc Exp Biol Med* 1968, **127**(3):820-823.

20. Ikeda R, Nishikawa A, Shinoda T, Fukazawa Y: **Chemical characterization of capsular polysaccharide from *Cryptococcus neoformans* serotype A-D.** *Microbiol Immunol* 1985, **29**(10):981-991.

21. Kwon-Chung KJ, Varma A: **Do major species concepts support one, two or more species within *Cryptococcus neoformans*?** *FEMS Yeast Res* 2006, **6**(4):574-587.

22. Vanbreuseghem R, Takashio M: **An atypical strain of *Cryptococcus neoformans* (San Felice) Vuillemin 1894. II. *Cryptococcus neoformans* var. *gattii* var. nov.** *Ann Soc Belges Med Trop Parasitol Mycol* 1970, **50**(6):695-702.

23. Kwon-Chung J, Boekhout T, Fell JW, Diaz M: **Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (*Basidiomycota, Hymenomycetes, Tremellomycetidae*).** *Taxon* 202, **51**:804 - 806.

24. Ngamskulrungroj P, Gilgado F, Faganello J, Litvintseva AP, Leal AL, Tsui KM, Mitchell TG, Vainstein MH, Meyer W: **Genetic diversity of the *Cryptococcus***

species complex suggests that *Cryptococcus gattii* deserves to have varieties. *PLoS One* 2009, **4**(6):e5862.

25. Boekhout T, van Belkum A, Leenders AC, Verbrugh HA, Mukamurangwa P, Swinne D, Scheffers WA: **Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects.** *Int J Syst Bacteriol* 1997, **47**(2):432-442.

26. Chen SC, Brownlee AG, Sorrell TC, Ruma P, Nimmo G: **Identification by random amplification of polymorphic DNA of a common molecular type of *Cryptococcus neoformans* var. *neoformans* in patients with AIDS or other immunosuppressive conditions.** *J Infect Dis* 1996, **173**(3):754-758.

27. Lane M, McBride J, Archer J: **Steroid responsive late deterioration in *Cryptococcus neoformans* variety *gattii* meningitis.** *Neurology* 2004, **63**(4):713-714.

28. Meyer W, Mitchell TG, Freedman EZ, Vilgalys R: **Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*.** *J Clin Microbiol* 1993, **31**(9):2274-2280.

29. Phillips P, Chapman K, Sharp M, Harrison P, Vortel J, Steiner T, Bowie W: **Dexamethasone in *Cryptococcus gattii* central nervous system infection.** *Clin Infect Dis* 2009, **49**(4):591-595.

30. Ruma P, Chen SC, Sorrell TC, Brownlee AG: **Characterization of *Cryptococcus neoformans* by random DNA amplification.** *Lett Appl Microbiol* 1996, **23**(5):312-316.

31. Seaton RA, Verma N, Naraqi S, Wembri JP, Warrell DA: **The effect of corticosteroids on visual loss in *Cryptococcus neoformans* var. *gattii* meningitis**. *Trans R Soc Trop Med Hyg* 1997, **91**(1):50-52.

32. Sorrell TC, Chen SC, Ruma P, Meyer W, Pfeiffer TJ, Ellis DH, Brownlee AG: **Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting**. *J Clin Microbiol* 1996, **34**(5):1253-1260.

33. Wickes BL, Moore TD, Kwon-Chung KJ: **Comparison of the electrophoretic karyotypes and chromosomal location of ten genes in the two varieties of *Cryptococcus neoformans***. *Microbiology* 1994, **140 (Pt 3)**:543-550.

34. Boekhout T, Theelen B, Diaz M, Fell JW, Hop WC, Abeln EC, Dromer F, Meyer W: **Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans***. *Microbiology* 2001, **147**(Pt 4):891-907.

35. Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E: **Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates**. *Emerg Infect Dis* 2003, **9**(2):189-195.

36. Franzot SP, Salkin IF, Casadevall A: ***Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates**. *JClinMicrobiol* 1999, **37**(3):838-840.

37. Lengeler KB, Wang P, Cox GM, Perfect JR, Heitman J: **Identification of the MATa mating-type locus of *Cryptococcus neoformans* reveals a serotype A MATa strain thought to have been extinct**. *Proc Natl Acad Sci U S A* 2000, **97**(26):14455-14460.

38. Nielsen K, Cox GM, Litvintseva AP, Mylonakis E, Malliaris SD, Benjamin DK, Jr., Giles SS, Mitchell TG, Casadevall A, Perfect JR *et al*: ***Cryptococcus neoformans*** **{alpha} strains preferentially disseminate to the central nervous system during coinfection**. *Infect Immun* 2005, **73**(8):4922-4933.

39. Tscharke RL, Lazera M, Chang YC, Wickes BL, Kwon-Chung KJ: **Haploid fruiting in *Cryptococcus neoformans* is not mating type alpha-specific**. *Fungal Genet Biol* 2003, **39**(3):230-237.

40. Ellis DH, Pfeiffer TJ: **Ecology, life cycle, and infectious propagule of *Cryptococcus neoformans***. *Lancet* 1990, **336**(8720):923-925.

41. Cruz MC, Fox DS, Heitman J: **Calcineurin is required for hyphal elongation during mating and haploid fruiting in *Cryptococcus neoformans***. *Embo J* 2001, **20**(5):1020-1032.

42. Khan ZU, Pal M, Randhawa HS, Sandhu RS: **Carriage of *Cryptococcus neoformans* in the crops of pigeons**. *J Med Microbiol* 1978, **11**(2):215-218.

43. Khosravi AR: **Isolation of *Cryptococcus neoformans* from pigeon (Columbia livia droppings in northern Iran**. *Mycopathologia* 1997, **139**(2):93-95.

44. Schonheyder H, Stenderup A: **Isolation of *Cryptococcus neoformans* from pigeon manure on two media inducing pigment formation**. *Sabouraudia* 1982, **20**(3):193-197.

45. Yamamoto Y, Kohno S, Noda T, Kakeya H, Yanagihara K, Ohno H, Ogawa K, Kawamura S, Ohtsubo T, Tomono K *et al*: **[Isolation of *Cryptococcus neoformans* from environments (pigeon excreta) in Nagasaki]**. *Kansenshogaku Zasshi* 1995, **69**(6):642-645.

46. Emmons CW: **Isolation of *Cryptococcus neoformans* from soil.** *J Bacteriol* 1951, **62**(6):685-690.

47. Lazera MS, Pires FD, Camillo-Coura L, Nishikawa MM, Bezerra CC, Trilles L, Wanke B: **Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees.** *J Med Vet Mycol* 1996, **34**(2):127-131.

48. Ellis DH, Pfeiffer TJ: **Natural habitat of *Cryptococcus neoformans* var. *gattii*.** *J Clin Microbiol* 1990, **28**(7):1642-1644.

49. Kidd SE, Chow Y, Mak S, Bach PJ, Chen H, Hingston AO, Kronstad JW, Bartlett KH: **Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States.** *Appl Environ Microbiol* 2007, **73**(5):1433-1443.

50. Gugnani HC, Mitchell TG, Litvintseva AP, Lengeler KB, Heitman J, Kumar A, Basu S, Paliwal-Joshi A: **Isolation of *Cryptococcus gattii* and *Cryptococcus neoformans* var. *grubii* from the flowers and bark of Eucalyptus trees in India.** *Med Mycol* 2005, **43**(6):565-569.

51. DeShaw M, Pirofski LA: **Antibodies to the *Cryptococcus neoformans* capsular glucuronoxylomannan are ubiquitous in serum from HIV+ and HIV- individuals.** *Clin Exp Immunol* 1995, **99**(3):425-432.

52. Friedman GD: **The rarity of cryptococcosis in Northern California: the 10-year experience of a large defined population.** *Am J Epidemiol* 1983, **117**(2):230-234.

53. Sun HY, Wagener MM, Singh N: **Cryptococcosis in solid-organ, hematopoietic stem cell, and tissue transplant recipients: evidence-based evolving trends**. *Clin Infect Dis* 2009, **48**(11):1566-1576.

54. Munoz P, Giannella M, Valerio M, Soria T, Diaz F, Longo JL, Bouza E: **Cryptococcal meningitis in a patient treated with infliximab**. *Diagn Microbiol Infect Dis* 2007, **57**(4):443-446.

55. Tuxen AJ, Yong MK, Street AC, Dolianitis C: **Disseminated cryptococcal infection in a patient with severe psoriasis treated with efalizumab, methotrexate and ciclosporin**. *Br J Dermatol* 2007, **157**(5):1067-1068.

56. WHO: **Revised Global Burden of Disease (GBD) 2002 Estimates.** In.; 2002.

57. French N, Gray K, Watera C, Nakiyingi J, Lugada E, Moore M, Lalloo D, Whitworth JA, Gilks CF: **Cryptococcal infection in a cohort of HIV-1-infected Ugandan adults**. *AIDS* 2002, **16**(7):1031-1038.

58. Louie JK, Chi NH, Thao le TT, Quang VM, Campbell J, Chau NV, Rutherford GW, Farrar JJ, Parry CM: **Opportunistic infections in hospitalized HIV-infected adults in Ho Chi Minh City, Vietnam: a cross-sectional study**. *Int J STD AIDS* 2004, **15**(11):758-761.

59. Wong ML, Back P, Candy G, Nelson G, Murray J: **Cryptococcal pneumonia in African miners at autopsy**. *Int J Tuberc Lung Dis* 2007, **11**(5):528-533.

60. Laurenson IF, Trevett AJ, Lalloo DG, Nwokolo N, Naraqi S, Black J, Tefurani N, Saweri A, Mavo B, Igo J *et al*: **Meningitis caused by *Cryptococcus neoformans* var. *gattii* and var. *neoformans* in Papua New Guinea**. *Trans R Soc Trop Med Hyg* 1996, **90**(1):57-60.

61. Mitchell DH, Sorrell TC, Allworth AM, Heath CH, McGregor AR, Papanaoum K, Richards MJ, Gottlieb T: **Cryptococcal disease of the CNS in immunocompetent hosts: influence of cryptococcal variety on clinical manifestations and outcome**. *ClinInfectDis* 1995, **20**(3):611-616.

62. Seaton RA, Naraqi S, Wembri JP, Warrell DA: **Predictors of outcome in *Cryptococcus neoformans* var. *gattii* meningitis**. *QJM* 1996, **89**(6):423-428.

63. Kwon-Chung KJ, Bennett JE: **High prevalence of *Cryptococcus neoformans* var. *gattii* in tropical and subtropical regions**. *Zentralbl Bakteriol Mikrobiol Hyg A* 1984, **257**(2):213-218.

64. Chen S, Sorrell T, Nimmo G, Speed B, Currie B, Ellis D, Marriott D, Pfeiffer T, Parr D, Byth K: **Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus neoformans* in Australia and New Zealand. Australasian Cryptococcal Study Group**. *ClinInfectDis* 2000, **31**(2):499-508.

65. Bartlett KH, Kidd SE, Kronstad JW: **The Emergence of *Cryptococcus gattii* in British Columbia and the Pacific Northwest**. *Curr Infect Dis Rep* 2008, **10**(1):58-65.

66. Duncan C, Schwantje H, Stephen C, Campbell J, Bartlett K: ***Cryptococcus gattii* in wildlife of Vancouver Island, British Columbia, Canada**. *J Wildl Dis* 2006, **42**(1):175-178.

67. Hoang LM, Maguire JA, Doyle P, Fyfe M, Roscoe DL: ***Cryptococcus neoformans* infections at Vancouver Hospital and Health Sciences Centre**

(1997-2002): epidemiology, microbiology and histopathology. *J Med Microbiol* 2004, **53**(Pt 9):935-940.

68. Khan ZU, Randhawa HS, Kowshik T, Chowdhary A, Chandy R: **Antifungal susceptibility of *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from decayed wood of trunk hollows of Ficus religiosa and Syzygium cumini trees in north-western India**. *J Antimicrob Chemother* 2007, **60**(2):312-316.

69. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, Macdougall L, Boekhout T, Kwon-Chung KJ, Meyer W: **A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada)**. *Proc Natl Acad Sci U S A* 2004, **101**(49):17258-17263.

70. Morgan J, McCarthy KM, Gould S, Fan K, Arthington-Skaggs B, Iqbal N, Stamey K, Hajjeh RA, Brandt ME: ***Cryptococcus gattii* infection: characteristics and epidemiology of cases identified in a South African province with high HIV seroprevalence, 2002-2004**. *Clin Infect Dis* 2006, **43**(8):1077-1080.

71. Krockenberger MB, Canfield PJ, Barnes J, Vogelnest L, Connolly J, Ley C, Malik R: ***Cryptococcus neoformans* var. *gattii* in the koala (Phascolarctos cinereus): serological evidence for subclinical cryptococcosis**. *Med Mycol* 2002, **40**(3):273-282.

72. Miller WG, Padhye AA, van Bonn W, Jensen E, Brandt ME, Ridgway SH: **Cryptococcosis in a bottlenose dolphin (*Tursiops truncatus*) caused by *Cryptococcus neoformans* var. *gattii***. *J Clin Microbiol* 2002, **40**(2):721-724.

73. MacDougall L, Fyfe M: **Emergence of *Cryptococcus gattii* in a novel environment provides clues to its incubation period.** *J Clin Microbiol* 2006, **44**(5):1851-1852.

74. Garcia-Hermoso D, Janbon G, Dromer F: **Epidemiological evidence for dormant *Cryptococcus neoformans* infection.** *J Clin Microbiol* 1999, **37**(10):3204-3209.

75. Fessel WJ: **Cryptococcal meningitis after unusual exposures to birds.** *N Engl J Med* 1993, **328**(18):1354-1355.

76. Speed B, Dunt D: **Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans*.** *Clin Infect Dis* 1995, **21**(1):28-34; discussion 35-26.

77. Seaton RA, Verma N, Naraqi S, Wembri JP, Warrell DA: **The effect of corticosteroids on visual loss in *Cryptococcus neoformans* var. *gattii* meningitis.** *TransRSocTropMedHyg* 1997, **91**(1):50-52.

78. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH *et al*: **Clinical Practice Guidelines for the Management of Cryptococcal Disease: 2010 Update by the Infectious Diseases Society of America.** *Clin Infect Dis* 2010.

79. Thwaites GE, Chau TT, Stepniewska K, Phu NH, Chuong LV, Sinh DX, White NJ, Parry CM, Farrar JJ: **Diagnosis of adult tuberculous meningitis by use of clinical and laboratory features.** *Lancet* 2002, **360**(9342):1287-1292.

80. Seaton RA, Verma N, Naraqi S, Wembri JP, Warrell DA: **Visual loss in immunocompetent patients with *Cryptococcus neoformans* var. *gattii* meningitis**. *TransRSocTropMedHyg* 1997, **91**(1):44-49.

81. Thwaites GE, Duc Bang N, Huy Dung N, Thi Quy H, Thi Tuong Oanh D, Thi Cam Thoa N, Quang Hien N, Tri Thuc N, Ngoc Hai N, Thi Ngoc Lan N *et al*: **The influence of HIV infection on clinical presentation, response to treatment, and outcome in adults with Tuberculous meningitis**. *J Infect Dis* 2005, **192**(12):2134-2141.

82. Mansour A, Nakhla I, El Sherif M, Sultan YA, Frenck RW: ***Cryptococcus neoformans* var. *gattii* meningitis in Egypt: a case report**. *East Mediterr Health J* 2006, **12**(1-2):241-244.

83. Bodasing N, Seaton RA, Shankland GS, Kennedy D: ***Cryptococcus neoformans* var. *gattii* meningitis in an HIV-positive patient: first observation in the United Kingdom**. *J Infect* 2004, **49**(3):253-255.

84. Karstaedt AS, Crewe-Brown HH, Dromer F: **Cryptococcal meningitis caused by *Cryptococcus neoformans* var. *gattii*, serotype C, in AIDS patients in Soweto, South Africa**. *Med Mycol* 2002, **40**(1):7-11.

85. Bogaerts J, Rouvroy D, Taelman H, Kagame A, Aziz MA, Swinne D, Verhaegen J: **AIDS-associated cryptococcal meningitis in Rwanda (1983-1992): epidemiologic and diagnostic features**. *J Infect* 1999, **39**(1):32-37.

86. Seaton RA, Wembri JP, Armstrong P, Ombiga J, Naraqi S, Kevau I: **Symptomatic human immunodeficiency virus (HIV) infection in Papua New Guinea**. *Aust N Z J Med* 1996, **26**(6):783-788.

87. Bogaerts J, Taelman H, Batungwanayo J, Van de Perre P, Swinne D: **Two cases of HIV-associated cryptococcosis due to the variety *gattii* in Rwanda**. *Trans R Soc Trop Med Hyg* 1993, **87**(1):63-64.

88. Kapend'a K, Komichelo K, Swinne D, Vandepitte J: **Meningitis due to *Cryptococcus neoformans* biovar *gattii* in a Zairean AIDS patient**. *Eur J Clin Microbiol* 1987, **6**(3):320-321.

89. Swinne D, Nkurikiyinfura JB, Muyembe TL: **Clinical isolates of *Cryptococcus neoformans* from Zaire**. *Eur J Clin Microbiol* 1986, **5**(1):50-51.

90. Vender JR, Miller DM, Roth T, Nair S, Reboli AC: **Intraventricular cryptococcal cysts**. *AJNR Am J Neuroradiol* 1996, **17**(1):110-113.

91. Kovoor JM, Mahadevan A, Narayan JP, Govindappa SS, Satishchandra P, Taly AV, Shankar SK: **Cryptococcal choroid plexitis as a mass lesion: MR imaging and histopathologic correlation**. *AJNR Am J Neuroradiol* 2002, **23**(2):273-276.

92. Aharon-Peretz J, Kliot D, Finkelstein R, Ben Hayun R, Yarnitsky D, Goldsher D: **Cryptococcal meningitis mimicking vascular dementia**. *Neurology* 2004, **62**(11):2135.

93. Thwaites GE, Chau TT, Farrar JJ: **Improving the bacteriological diagnosis of tuberculous meningitis**. *J Clin Microbiol* 2004, **42**(1):378-379.

94. Wu TC, Koo SY: **Comparison of three commercial cryptococcal latex kits for detection of cryptococcal antigen**. *J Clin Microbiol* 1983, **18**(5):1127-1130.

95. Tanner DC, Weinstein MP, Fedorciw B, Joho KL, Thorpe JJ, Reller L: **Comparison of commercial kits for detection of cryptococcal antigen.** *J Clin Microbiol* 1994, **32**(7):1680-1684.

96. Feldmesser M, Harris C, Reichberg S, Khan S, Casadevall A: **Serum cryptococcal antigen in patients with AIDS.** *Clin Infect Dis* 1996, **23**(4):827-830.

97. Perfect JR, Casadevall A: **Cryptococcosis.** *Infect Dis Clin North Am* 2002, **16**(4):837-874, v-vi.

98. Cecchini D, Ambrosioni J, Brezzo C, Corti M, Rybko A, Perez M, Poggi S, Ambroggi M: **Tuberculous meningitis in HIV-infected patients: drug susceptibility and clinical outcome.** *Aids* 2007, **21**(3):373-374.

99. Brouwer AE, Rajanuwong A, Chierakul W, Griffin GE, Larsen RA, White NJ, Harrison TS: **Combination antifungal therapies for HIV-associated cryptococcal meningitis: a randomised trial.** *Lancet* 2004, **363**(9423):1764-1767.

100. Chuck SL, Sande MA: **Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome.** *NEnglJMed* 1989, **321**(12):794-799.

101. Saag MS, Powderly WG, Cloud GA, Robinson P, Grieco MH, Sharkey PK, Thompson SE, Sugar AM, Tuazon CU, Fisher JF *et al*: **Comparison of amphotericin B with fluconazole in the treatment of acute AIDS-associated cryptococcal meningitis. The NIAID Mycoses Study Group and the AIDS Clinical Trials Group.** *NEnglJMed* 1992, **326**(2):83-89.

102. Dromer F, Mathoulin S, Dupont B, Brugiere O, Letenneur L: **Comparison of the efficacy of amphotericin B and fluconazole in the treatment of cryptococcosis in human immunodeficiency virus-negative patients: retrospective analysis of 83 cases. French Cryptococcosis Study Group.** *ClinInfectDis* 1996, **22 Suppl 2**:S154-S160.

103. Lu CH, Chang WN, Chang HW, Chuang YC: **The prognostic factors of cryptococcal meningitis in HIV-negative patients.** *JHospInfect* 1999, **42**(4):313-320.

104. Lui G, Lee N, Ip M, Choi KW, Tso YK, Lam E, Chau S, Lai R, Cockram CS: **Cryptococcosis in apparently immunocompetent patients.** *Qjm* 2006, **99**(3):143-151.

105. Maher D, Mwandumba H: **Cryptococcal meningitis in Lilongwe and Blantyre, Malawi.** *J Infect* 1994, **28**(1):59-64.

106. Pitisuttithum P, Tansuphasawadikul S, Simpson AJ, Howe PA, White NJ: **A prospective study of AIDS-associated cryptococcal meningitis in Thailand treated with high-dose amphotericin B.** *JInfect* 2001, **43**(4):226-233.

107. van der Horst CM, Saag MS, Cloud GA, Hamill RJ, Graybill JR, Sobel JD, Johnson PC, Tuazon CU, Kerkering T, Moskovitz BL *et al*: **Treatment of cryptococcal meningitis associated with the acquired immunodeficiency syndrome. National Institute of Allergy and Infectious Diseases Mycoses Study Group and AIDS Clinical Trials Group.** *NEnglJMed* 1997, **337**(1):15-21.

108. Graybill JR, Sobel J, Saag M, van Der HC, Powderly W, Cloud G, Riser L, Hamill R, Dismukes W: **Diagnosis and management of increased intracranial pressure in patients with AIDS and cryptococcal meningitis. The NIAID Mycoses Study Group and AIDS Cooperative Treatment Groups**. *ClinInfectDis* 2000, **30**(1):47-54.

109. Newton PN, Thai le H, Tip NQ, Short JM, Chierakul W, Rajanuwong A, Pitisuttithum P, Chasombat S, Phonrat B, Maek ANW *et al*: **A randomized, double-blind, placebo-controlled trial of acetazolamide for the treatment of elevated intracranial pressure in cryptococcal meningitis**. *Clin Infect Dis* 2002, **35**(6):769-772.

110. Saag MS, Graybill RJ, Larsen RA, Pappas PG, Perfect JR, Powderly WG, Sobel JD, Dismukes WE: **Practice guidelines for the management of cryptococcal disease. Infectious Diseases Society of America**. *ClinInfectDis* 2000, **30**(4):710-718.

111. Chang WN, Huang CR, Lei CB, Lee PY, Chien CC, Chang HW, Chang CS, Lu CH: **Serotypes of clinical cerebrospinal fluid *Cryptococcus neoformans* isolates from southern Taiwan and their in vitro susceptibilities to amphotericin B, fluconazole, and voriconazole**. *Jpn J Infect Dis* 2004, **57**(3):113-115.

112. Chandenier J, Adou-Bryn KD, Douchet C, Sar B, Kombila M, Swinne D, Therizol-Ferly M, Buisson Y, Richard-Lenoble D: **In vitro activity of amphotericin B, fluconazole and voriconazole against 162 *Cryptococcus***

***neoformans* isolates from Africa and Cambodia**. *Eur J Clin Microbiol Infect Dis* 2004, **23**(6):506-508.

113. Lozano-Chiu M, Paetznick VL, Ghannoum MA, Rex JH: **Detection of resistance to amphotericin B among *Cryptococcus neoformans* clinical isolates: performances of three different media assessed by using E-test and National Committee for Clinical Laboratory Standards M27-A methodologies**. *J Clin Microbiol* 1998, **36**(10):2817-2822.

114. Arechavala AI, Ochiuzzi ME, Borgnia MD, Santiso GM: **Fluconazole and amphotericin B susceptibility testing of *Cryptococcus neoformans*: Results of minimal inhibitory concentrations against 265 isolates from HIV-positive patients before and after two or more months of antifungal therapy**. *Rev Iberoam Micol* 2009, **26**(3):194-197.

115. Kantarcioglu AS, Boekhout T, Yucel A, Altas K: **Susceptibility testing of *Cryptococcus diffluens* against amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole and posaconazole**. *Med Mycol* 2009, **47**(2):169-176.

116. Coste A, Linas MD, Cassaing S, Bernad J, Chalmeton S, Seguela JP, Pipy B: **A sub-inhibitory concentration of amphotericin B enhances candidastatic activity of interferon-gamma- and interleukin-13-treated murine peritoneal macrophages**. *J Antimicrob Chemother* 2002, **49**(5):731-740.

117. Reyes E, Cardona J, Prieto A, Bernstein ED, Rodriguez-Zapata M, Pontes MJ, Alvarez-Mon M: **Liposomal amphotericin B and amphotericin B-deoxycholate show different immunoregulatory effects on human peripheral blood mononuclear cells**. *J Infect Dis* 2000, **181**(6):2003-2010.

118. Tohyama M, Kawakami K, Saito A: **Anticryptococcal effect of amphotericin B is mediated through macrophage production of nitric oxide.** *Antimicrob Agents Chemother* 1996, **40**(8):1919-1923.

119. Khoo SH, Bond J, Denning DW: **Administering amphotericin B--a practical approach.** *JAntimicrobChemother* 1994, **33**(2):203-213.

120. Schwarz P, Dromer F, Lortholary O, Dannaoui E: **In vitro interaction of flucytosine with conventional and new antifungals against *Cryptococcus neoformans* clinical isolates.** *Antimicrob Agents Chemother* 2003, **47**(10):3361-3364.

121. Vermes A, Guchelaar HJ, Dankert J: **Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions.** *J Antimicrob Chemother* 2000, **46**(2):171-179.

122. Tucker RM, Williams PL, Arathoon EG, Levine BE, Hartstein AI, Hanson LH, Stevens DA: **Pharmacokinetics of fluconazole in cerebrospinal fluid and serum in human coccidioidal meningitis.** *Antimicrob Agents Chemother* 1988, **32**(3):369-373.

123. Rex JH, Pappas PG, Karchmer AW, Sobel J, Edwards JE, Hadley S, Brass C, Vazquez JA, Chapman SW, Horowitz HW *et al*: **A randomized and blinded multicenter trial of high-dose fluconazole plus placebo versus fluconazole plus amphotericin B as therapy for candidemia and its consequences in nonneutropenic subjects.** *Clin Infect Dis* 2003, **36**(10):1221-1228.

124. Torres-Rodriguez JM, Alvarado-Ramirez E, Murciano F, Sellart M: **MICs and minimum fungicidal concentrations of posaconazole, voriconazole and**

fluconazole for *Cryptococcus neoformans* and *Cryptococcus gattii*. *J Antimicrob Chemother* 2008, **62**(1):205-206.

125. Sucher AJ, Chahine EB, Balcer HE: **Echinocandins: the newest class of antifungals**. *Ann Pharmacother* 2009, **43**(10):1647-1657.

126. Siddiqui AA, Brouwer AE, Wuthiekanun V, Jaffar S, Shattock R, Irving D, Sheldon J, Chierakul W, Peacock S, Day N *et al*: **IFN-gamma at the site of infection determines rate of clearance of infection in cryptococcal meningitis**. *J Immunol* 2005, **174**(3):1746-1750.

127. Pappas PG, Bustamante B, Ticona E, Hamill RJ, Johnson PC, Reboli A, Aberg J, Hasbun R, Hsu HH: **Recombinant interferon- gamma 1b as adjunctive therapy for AIDS-related acute cryptococcal meningitis**. *J Infect Dis* 2004, **189**(12):2185-2191.

128. Simmons CP, Thwaites GE, Quyen NT, Chau TT, Mai PP, Dung NT, Stepniewska K, White NJ, Hien TT, Farrar J: **The clinical benefit of adjunctive dexamethasone in tuberculous meningitis is not associated with measurable attenuation of peripheral or local immune responses**. *J Immunol* 2005, **175**(1):579-590.

129. Thwaites GE, Nguyen DB, Nguyen HD, Hoang TQ, Do TT, Nguyen TC, Nguyen QH, Nguyen TT, Nguyen NH, Nguyen TN *et al*: **Dexamethasone for the treatment of tuberculous meningitis in adolescents and adults**. *N Engl J Med* 2004, **351**(17):1741-1751.

130. Nguyen TH, Tran TH, Thwaites G, Ly VC, Dinh XS, Ho Dang TN, Dang QT, Nguyen DP, Nguyen HP, To SD *et al*: **Dexamethasone in Vietnamese**

adolescents and adults with bacterial meningitis. *N Engl J Med* 2007, **357**(24):2431-2440.

131. Scarborough M, Gordon SB, Whitty CJ, French N, Njalale Y, Chitani A, Peto TE, Lalloo DG, Zijlstra EE: **Corticosteroids for bacterial meningitis in adults in sub-Saharan Africa**. *N Engl J Med* 2007, **357**(24):2441-2450.

132. de Gans J, van de Beek D: **Dexamethasone in adults with bacterial meningitis**. *N Engl J Med* 2002, **347**(20):1549-1556.

133. Lawn SD, Harries AD, Anglaret X, Myer L, Wood R: **Early mortality among adults accessing antiretroviral treatment programmes in sub-Saharan Africa**. *Aids* 2008, **22**(15):1897-1908.

134. Zolopa A, Andersen J, Powderly W, Sanchez A, Sanne I, Suckow C, Hogg E, Komarow L: **Early antiretroviral therapy reduces AIDS progression/death in individuals with acute opportunistic infections: a multicenter randomized strategy trial**. *PLoS One* 2009, **4**(5):e5575.

135. Manosuthi W, Chottanapund S, Sungkanuparph S: **Mortality rate of early versus deferred initiation of antiretroviral therapy in HIV-1-infected patients with cryptococcal meningitis**. *J Acquir Immune Defic Syndr* 2008, **48**(4):508-509.

136. Makadzange AT, Ndhlovu CE, Takarinda K, Reid M, Kurangwa M, Gona P, Hakim JG: **Early versus delayed initiation of antiretroviral therapy for concurrent HIV infection and cryptococcal meningitis in sub-saharan Africa**. *Clin Infect Dis*, **50**(11):1532-1538.

137. Bicanic T, Wood R, Meintjes G, Rebe K, Brouwer A, Loyse A, Bekker LG, Jaffar S, Harrison T: **High-dose amphotericin B with flucytosine for the treatment of cryptococcal meningitis in HIV-infected patients: a randomized trial**. *Clin Infect Dis* 2008, **47**(1):123-130.

138. Bicanic T, Muzoora C, Brouwer AE, Meintjes G, Longley N, Taseera K, Rebe K, Loyse A, Jarvis J, Bekker LG *et al*: **Independent association between rate of clearance of infection and clinical outcome of HIV-associated cryptococcal meningitis: analysis of a combined cohort of 262 patients**. *Clin Infect Dis* 2009, **49**(5):702-709.

139. Dromer F, Bernede-Bauduin C, Guillemot D, Lortholary O: **Major role for amphotericin B-flucytosine combination in severe cryptococcosis**. *PLoS One* 2008, **3**(8):e2870.

140. Bates DW, Su L, Yu DT, Chertow GM, Seger DL, Gomes DR, Dasbach EJ, Platt R: **Mortality and costs of acute renal failure associated with amphotericin B therapy**. *Clin Infect Dis* 2001, **32**(5):686-693.

141. Bates DW, Su L, Yu DT, Chertow GM, Seger DL, Gomes DR, Platt R: **Correlates of acute renal failure in patients receiving parenteral amphotericin B**. *Kidney Int* 2001, **60**(4):1452-1459.

142. Larsen RA, Leal MA, Chan LS: **Fluconazole compared with amphotericin B plus flucytosine for cryptococcal meningitis in AIDS. A randomized trial.** *AnnInternMed* 1990, **113**(3):183-187.

143. Menichetti F, Fiorio M, Tosti A, Gatti G, Bruna Pasticci M, Miletich F, Marroni M, Bassetti D, Pauluzzi S: **High-dose fluconazole therapy for cryptococcal meningitis in patients with AIDS**. *Clin Infect Dis* 1996, **22**(5):838-840.

144. Mayanja-Kizza H, Oishi K, Mitarai S, Yamashita H, Nalongo K, Watanabe K, Izumi T, Ococi J, Augustine K, Mugerwa R *et al*: **Combination therapy with fluconazole and flucytosine for cryptococcal meningitis in Ugandan patients with AIDS**. *ClinInfectDis* 1998, **26**(6):1362-1366.

145. Milefchik E, Leal MA, Haubrich R, Bozzette SA, Tilles JG, Leedom JM, McCutchan JA, Larsen RA: **Fluconazole alone or combined with flucytosine for the treatment of AIDS-associated cryptococcal meningitis**. *Med Mycol* 2008, **46**(4):393-395.

146. de Gans J, Portegies P, Tiessens G, Eeftinck Schattenkerk JK, van Boxtel CJ, van Ketel RJ, Stam J: **Itraconazole compared with amphotericin B plus flucytosine in AIDS patients with cryptococcal meningitis**. *Aids* 1992, **6**(2):185-190.

147. Bozzette SA, Larsen RA, Chiu J, Leal MA, Jacobsen J, Rothman P, Robinson P, Gilbert G, McCutchan JA, Tilles J *et al*: **A placebo-controlled trial of maintenance therapy with fluconazole after treatment of cryptococcal meningitis in the acquired immunodeficiency syndrome. California Collaborative Treatment Group**. *N Engl J Med* 1991, **324**(9):580-584.

148. Powderly WG, Saag MS, Cloud GA, Robinson P, Meyer RD, Jacobson JM, Graybill JR, Sugar AM, McAuliffe VJ, Follansbee SE *et al*: **A controlled trial of fluconazole or amphotericin B to prevent relapse of cryptococcal**

meningitis in patients with the acquired immunodeficiency syndrome. The NIAID AIDS Clinical Trials Group and Mycoses Study Group. *N Engl J Med* 1992, **326**(12):793-798.

149. Saag MS, Cloud GA, Graybill JR, Sobel JD, Tuazon CU, Johnson PC, Fessel WJ, Moskovitz BL, Wiesinger B, Cosmatos D *et al*: **A comparison of itraconazole versus fluconazole as maintenance therapy for AIDS-associated cryptococcal meningitis. National Institute of Allergy and Infectious Diseases Mycoses Study Group.** *ClinInfectDis* 1999, **28**(2):291-296.

150. Vibhagool A, Sungkanuparph S, Mootsikapun P, Chetchotisakd P, Tansuphaswaswadikul S, Bowonwatanuwong C, Ingsathit A: **Discontinuation of secondary prophylaxis for cryptococcal meningitis in human immunodeficiency virus-infected patients treated with highly active antiretroviral therapy: a prospective, multicenter, randomized study.** *Clin Infect Dis* 2003, **36**(10):1329-1331.

151. Mussini C, Pezzotti P, Miro JM, Martinez E, de Quiros JC, Cinque P, Borghi V, Bedini A, Domingo P, Cahn P *et al*: **Discontinuation of maintenance therapy for cryptococcal meningitis in patients with AIDS treated with highly active antiretroviral therapy: an international observational study.** *Clin Infect Dis* 2004, **38**(4):565-571.

152. Vilchez RA, Fung J, Kusne S: **Cryptococcosis in organ transplant recipients: an overview.** *Am J Transplant* 2002, **2**(7):575-580.

153. Singh N, Lortholary O, Alexander BD, Gupta KL, John GT, Pursell KJ, Munoz P, Klintmalm GB, Stosor V, Del Busto R *et al*: **Antifungal management**

practices and evolution of infection in organ transplant recipients with *Cryptococcus neoformans* infection. *Transplantation* 2005, **80**(8):1033-1039.

154. Bennett JE, Dismukes WE, Duma RJ, Medoff G, Sande MA, Gallis H, Leonard J, Fields BT, Bradshaw M, Haywood H *et al*: **A comparison of amphotericin B alone and combined with flucytosine in the treatment of cryptococcal meningitis**. *NEnglJMed* 1979, **301**(3):126-131.

155. Dismukes WE, Cloud G, Gallis HA, Kerkering TM, Medoff G, Craven PC, Kaplowitz LG, Fisher JF, Gregg CR, Bowles CA *et al*: **Treatment of cryptococcal meningitis with combination amphotericin B and flucytosine for four as compared with six weeks**. *NEnglJMed* 1987, **317**(6):334-341.

156. Dromer F, Mathoulin-Pelissier S, Launay O, Lortholary O: **Determinants of disease presentation and outcome during cryptococcosis: the CryptoA/D study**. *PLoS Med* 2007, **4**(2):e21.

157. Pappas PG, Perfect JR, Cloud GA, Larsen RA, Pankey GA, Lancaster DJ, Henderson H, Kauffman CA, Haas DW, Saccente M *et al*: **Cryptococcosis in human immunodeficiency virus-negative patients in the era of effective azole therapy**. *Clin Infect Dis* 2001, **33**(5):690-699.

158. Morera-Lopez Y, Torres-Rodriguez JM, Jimenez-Cabello T, Baro-Tomas T, Alia-Aponte C, Lazera MS: **DNA fingerprinting pattern and susceptibility to antifungal drugs in *Cryptococcus neoformans* variety *grubii* isolates from Barcelona city and rural environmental samples**. *Mycopathologia* 2005, **160**(1):9-14.

159. Aller AI, Martin-Mazuelos E, Gutierrez MJ, Bernal S, Chavez M, Recio FJ: **Comparison of the Etest and microdilution method for antifungal susceptibility testing of *Cryptococcus neoformans* to four antifungal agents.** *J Antimicrob Chemother* 2000, **46**(6):997-1000.

160. Colombo AL, Barchiesi F, McGough DA, Rinaldi MG: **Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for azole antifungal susceptibility testing.** *J Clin Microbiol* 1995, **33**(3):535-540.

161. Dannaoui E, Abdul M, Arpin M, Michel-Nguyen A, Piens MA, Favel A, Lortholary O, Dromer F: **Results obtained with various antifungal susceptibility testing methods do not predict early clinical outcome in patients with cryptococcosis.** *Antimicrob Agents Chemother* 2006, **50**(7):2464-2470.

162. Davey KG, Holmes AD, Johnson EM, Szekely A, Warnock DW: **Comparative evaluation of FUNGITEST and broth microdilution methods for antifungal drug susceptibility testing of *Candida* species and *Cryptococcus neoformans*.** *J Clin Microbiol* 1998, **36**(4):926-930.

163. Davey KG, Szekely A, Johnson EM, Warnock DW: **Comparison of a new commercial colorimetric microdilution method with a standard method for in-vitro susceptibility testing of Candida spp. and *Cryptococcus neoformans*.** *J Antimicrob Chemother* 1998, **42**(4):439-444.

164. Dias AL, Matsumoto FE, Melhem MS, da Silva EG, Auler ME, de Siqueira AM, Paula CR: **Comparative analysis of Etest and broth microdilution method**

(AFST-EUCAST) for trends in antifungal drug susceptibility testing of Brazilian *Cryptococcus neoformans* isolates. *J Med Microbiol* 2006, **55**(Pt 12):1693-1699.

165. Espinel-Ingroff A: **Comparison of three commercial assays and a modified disk diffusion assay with two broth microdilution reference assays for testing zygomycetes, *Aspergillus* spp., *Candida* spp., and *Cryptococcus neoformans* with posaconazole and amphotericin B.** *J Clin Microbiol* 2006, **44**(10):3616-3622.

166. Espinel-Ingroff A, Barchiesi F, Cuenca-Estrella M, Pfaller MA, Rinaldi M, Rodriguez-Tudela JL, Verweij PE: **International and multicenter comparison of EUCAST and CLSI M27-A2 broth microdilution methods for testing susceptibilities of *Candida* spp. to fluconazole, itraconazole, posaconazole, and voriconazole.** *J Clin Microbiol* 2005, **43**(8):3884-3889.

167. Espinel-Ingroff A, Pfaller M, Messer SA, Knapp CC, Killian S, Norris HA, Ghannoum MA: **Multicenter comparison of the sensititre YeastOne Colorimetric Antifungal Panel with the National Committee for Clinical Laboratory standards M27-A reference method for testing clinical isolates of common and emerging *Candida* spp., *Cryptococcus* spp., and other yeasts and yeast-like organisms.** *J Clin Microbiol* 1999, **37**(3):591-595.

168. Espinel-Ingroff A, Canton E, Gibbs D, Wang A: **Correlation of Neo-Sensitabs tablet diffusion assay results on three different agar media with CLSI broth microdilution M27-A2 and disk diffusion M44-A results for testing susceptibilities of *Candida* spp. and *Cryptococcus neoformans* to**

amphotericin B, caspofungin, fluconazole, itraconazole, and voriconazole. *J Clin Microbiol* 2007, **45**(3):858-864.

169. NCCLS: **Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Second Edition**. In. Wayne; 2002.

170. Anaissie EJ, Karyotakis NC, Hachem R, Dignani MC, Rex JH, Paetznick V: **Correlation between in vitro and in vivo activity of antifungal agents against *Candida* species.** *J Infect Dis* 1994, **170**(2):384-389.

171. Pfaller MA, Diekema DJ, Rex JH, Espinel-Ingroff A, Johnson EM, Andes D, Chaturvedi V, Ghannoum MA, Odds FC, Rinaldi MG *et al*: **Correlation of MIC with outcome for *Candida* species tested against voriconazole: analysis and proposal for interpretive breakpoints.** *J Clin Microbiol* 2006, **44**(3):819-826.

172. Rex JH, Pfaller MA, Galgiani JN, Bartlett MS, Espinel-Ingroff A, Ghannoum MA, Lancaster M, Odds FC, Rinaldi MG, Walsh TJ *et al*: **Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards.** *Clin Infect Dis* 1997, **24**(2):235-247.

173. Costa M, Passos XS, Miranda AT, de Araujo RS, Paula CR, Silva Mdo R: **Correlation of in vitro itraconazole and fluconazole susceptibility with clinical outcome for patients with vulvovaginal candidiasis.** *Mycopathologia* 2004, **157**(1):43-47.

174. Cameron ML, Schell WA, Bruch S, Bartlett JA, Waskin HA, Perfect JR: **Correlation of in vitro fluconazole resistance of *Candida* isolates in relation to therapy and symptoms of individuals seropositive for human immunodeficiency virus type 1**. *Antimicrob Agents Chemother* 1993, **37**(11):2449-2453.

175. Rodriguez-Tudela JL, Martinez-Suarez JV, Dronda F, Laguna F, Chaves F, Valencia E: **Correlation of in-vitro susceptibility test results with clinical response: a study of azole therapy in AIDS patients**. *J Antimicrob Chemother* 1995, **35**(6):793-804.

176. Quereda C, Polanco AM, Giner C, Sanchez-Sousa A, Pereira E, Navas E, Fortun J, Guerrero A, Baquero F: **Correlation between in vitro resistance to fluconazole and clinical outcome of oropharyngeal candidiasis in HIV-infected patients**. *Eur J Clin Microbiol Infect Dis* 1996, **15**(1):30-37.

177. Kartsonis N, Killar J, Mixson L, Hoe CM, Sable C, Bartizal K, Motyl M: **Caspofungin susceptibility testing of isolates from patients with esophageal candidiasis or invasive candidiasis: relationship of MIC to treatment outcome**. *Antimicrob Agents Chemother* 2005, **49**(9):3616-3623.

178. Park BJ, Arthington-Skaggs BA, Hajjeh RA, Iqbal N, Ciblak MA, Lee-Yang W, Hairston MD, Phelan M, Plikaytis BD, Sofair AN *et al*: **Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome**. *Antimicrob Agents Chemother* 2006, **50**(4):1287-1292.

179. Kovacicova G, Krupova Y, Lovaszova M, Roidova A, Trupl J, Liskova A, Hanzen J, Milosovic P, Lamosova M, Macekova L *et al*: **Antifungal susceptibility of 262 bloodstream yeast isolates from a mixed cancer and non-cancer patient population: is there a correlation between in-vitro resistance to fluconazole and the outcome of fungemia?** *J Infect Chemother* 2000, **6**(4):216-221.

180. Lee SC, Fung CP, Huang JS, Tsai CJ, Chen KS, Chen HY, Lee N, See LC, Shieh WB: **Clinical correlates of antifungal macrodilution susceptibility test results for non-AIDS patients with severe Candida infections treated with fluconazole.** *Antimicrob Agents Chemother* 2000, **44**(10):2715-2718.

181. Wenisch C, Moore CB, Krause R, Presterl E, Pichna P, Denning DW: **Antifungal susceptibility testing of fluconazole by flow cytometry correlates with clinical outcome.** *J Clin Microbiol* 2001, **39**(7):2458-2462.

182. Manosuthi W, Sungkanuparph S, Thongyen S, Chumpathat N, Eampokalap B, Thawornwan U, Foongladda S: **Antifungal susceptibilities of *Cryptococcus neoformans* cerebrospinal fluid isolates and clinical outcomes of cryptococcal meningitis in HIV-infected patients with/without fluconazole prophylaxis.** *J Med Assoc Thai* 2006, **89**(6):795-802.

183. Aller AI, Martin-Mazuelos E, Lozano F, Gomez-Mateos J, Steele-Moore L, Holloway WJ, Gutierrez MJ, Recio FJ, Espinel-Ingroff A: **Correlation of fluconazole MICs with clinical outcome in cryptococcal infection.** *Antimicrob Agents Chemother* 2000, **44**(6):1544-1548.

184. Goldman D, Song X, Kitai R, Casadevall A, Zhao ML, Lee SC: ***Cryptococcus neoformans*** **induces macrophage inflammatory protein 1alpha (MIP-1alpha) and MIP-1beta in human microglia: role of specific antibody and soluble capsular polysaccharide**. *InfectImmun* 2001, **69**(3):1808-1815.

185. Brandt ME, Pfaller MA, Hajjeh RA, Hamill RJ, Pappas PG, Reingold AL, Rimland D, Warnock DW: **Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992 to 1994 and 1996 to 1998**. *Antimicrob Agents Chemother* 2001, **45**(11):3065-3069.

186. Bicanic T, Brouwer AE, Meintjes G, Rebe K, Limmathurotsakul D, Chierakul W, Teparrakkul P, Loyse A, White NJ, Wood R *et al*: **Relationship of cerebrospinal fluid pressure, fungal burden and outcome in patients with cryptococcal meningitis undergoing serial lumbar punctures**. *Aids* 2009, **23**(6):701-706.

187. Coplin WM, Avellino AM, Kim DK, Winn HR, Grady MS: **Bacterial meningitis associated with lumbar drains: a retrospective cohort study**. *J Neurol Neurosurg Psychiatry* 1999, **67**(4):468-473.

188. Macsween KF, Bicanic T, Brouwer AE, Marsh H, Macallan DC, Harrison TS: **Lumbar drainage for control of raised cerebrospinal fluid pressure in cryptococcal meningitis: case report and review**. *J Infect* 2005, **51**(4):e221-224.

189. Pappas PG: **Managing cryptococcal meningitis is about handling the pressure**. *Clin Infect Dis* 2005, **40**(3):480-482.

190. Woodworth GF, McGirt MJ, Williams MA, Rigamonti D: **The use of ventriculoperitoneal shunts for uncontrollable intracranial hypertension without ventriculomegally secondary to HIV-associated cryptococcal meningitis**. *Surg Neurol* 2005, **63**(6):529-531; discussion 531-522.

191. Park MK, Hospenthal DR, Bennett JE: **Treatment of hydrocephalus secondary to cryptococcal meningitis by use of shunting**. *Clin Infect Dis* 1999, **28**(3):629-633.

192. Rex JH, Larsen RA, Dismukes WE, Cloud GA, Bennett JE: **Catastrophic visual loss due to *Cryptococcus neoformans* meningitis**. *Medicine (Baltimore)* 1993, **72**(4):207-224.

193. Montejo M, Aguirrebengoa K, de la Prieta R, Ibanez de Maeztu JC, Onate J, Gonzalez de Zarate P: **Blindness in patients with cryptococcal meningitis and AIDS**. *Eur Neurol* 1995, **35**(4):239-240.

194. Lan SH, Chang WN, Lu CH, Lui CC, Chang HW: **Cerebral infarction in chronic meningitis: a comparison of tuberculous meningitis and cryptococcal meningitis**. *QJM* 2001, **94**(5):247-253.

195. Bicanic T, Harrison T, Niepieklo A, Dyakopu N, Meintjes G: **Symptomatic relapse of HIV-associated cryptococcal meningitis after initial fluconazole monotherapy: the role of fluconazole resistance and immune reconstitution**. *Clin Infect Dis* 2006, **43**(8):1069-1073.

196. Afghani B, Lieberman JM: **Paradoxical enlargement or development of intracranial tuberculomas during therapy: case report and review**. *Clin Infect Dis* 1994, **19**(6):1092-1099.

197. Sungkanuparph S, Filler SG, Chetchotisakd P, Pappas PG, Nolen TL, Manosuthi W, Anekthananon T, Morris MI, Supparatpinyo K, Kopetskie H *et al*: **Cryptococcal immune reconstitution inflammatory syndrome after antiretroviral therapy in AIDS patients with cryptococcal meningitis: a prospective multicenter study**. *Clin Infect Dis* 2009, **49**(6):931-934.

198. Lortholary O, Fontanet A, Memain N, Martin A, Sitbon K, Dromer F: **Incidence and risk factors of immune reconstitution inflammatory syndrome complicating HIV-associated cryptococcosis in France**. *Aids* 2005, **19**(10):1043-1049.

199. Putignani L, Antonucci G, Paglia MG, Vincenzi L, Festa A, De Mori P, Loiacono L, Visca P: **Cryptococcal lymphadenitis as a manifestation of immune reconstitution inflammatory syndrome in an HIV-positive patient: a case report and review of the literature**. *Int J Immunopathol Pharmacol* 2008, **21**(3):751-756.

200. Trevenzoli M, Cattelan AM, Rea F, Sasset L, Semisa M, Lanzafame M, Meneghetti F, Cadrobi P: **Mediastinitis due to cryptococcal infection: a new clinical entity in the HAART era.** *J Infect* 2002, **45**(3):173-179.

201. Jarvis JN, Lawn SD, Vogt M, Bangani N, Wood R, Harrison TS: **Screening for cryptococcal antigenemia in patients accessing an antiretroviral treatment program in South Africa**. *Clin Infect Dis* 2009, **48**(7):856-862.

202. Imwidthaya P, Poungvarin N: **Cryptococcosis in AIDS**. *Postgrad Med J* 2000, **76**(892):85-88.

203. Lortholary O, Poizat G, Zeller V, Neuville S, Boibieux A, Alvarez M, Dellamonica P, Botterel F, Dromer F, Chene G: **Long-term outcome of AIDS-associated cryptococcosis in the era of combination antiretroviral therapy**. *Aids* 2006, **20**(17):2183-2191.

204. Kambugu A, Meya DB, Rhein J, O'Brien M, Janoff EN, Ronald AR, Kamya MR, Mayanja-Kizza H, Sande MA, Bohjanen PR *et al*: **Outcomes of cryptococcal meningitis in Uganda before and after the availability of highly active antiretroviral therapy**. *Clin Infect Dis* 2008, **46**(11):1694-1701.

205. Bicanic T, Meintjes G, Wood R, Hayes M, Rebe K, Bekker LG, Harrison T: **Fungal burden, early fungicidal activity, and outcome in cryptococcal meningitis in antiretroviral-naive or antiretroviral-experienced patients treated with amphotericin B or fluconazole**. *Clin Infect Dis* 2007, **45**(1):76-80.

206. Liechty CA, Solberg P, Were W, Ekwaru JP, Ransom RL, Weidle PJ, Downing R, Coutinho A, Mermin J: **Asymptomatic serum cryptococcal antigenemia and early mortality during antiretroviral therapy in rural Uganda**. *Trop Med Int Health* 2007, **12**(8):929-935.

207. Micol R, Lortholary O, Sar B, Laureillard D, Ngeth C, Dousset JP, Chanroeun H, Ferradini L, Guerin PJ, Dromer F *et al*: **Prevalence, determinants of positivity, and clinical utility of cryptococcal antigenemia in Cambodian HIV-infected patients**. *J Acquir Immune Defic Syndr* 2007, **45**(5):555-559.

208. McKinsey DS, Wheat LJ, Cloud GA, Pierce M, Black JR, Bamberger DM, Goldman M, Thomas CJ, Gutsch HM, Moskovitz B *et al*: **Itraconazole prophylaxis for fungal infections in patients with advanced human**

immunodeficiency virus infection: randomized, placebo-controlled, double-blind study. National Institute of Allergy and Infectious Diseases Mycoses Study Group. *Clin Infect Dis* 1999, **28**(5):1049-1056.

209. Chariyalertsak S, Supparatpinyo K, Sirisanthana T, Nelson KE: **A controlled trial of itraconazole as primary prophylaxis for systemic fungal infections in patients with advanced human immunodeficiency virus infection in Thailand**. *Clin Infect Dis* 2002, **34**(2):277-284.

210. Chetchotisakd P, Sungkanuparph S, Thinkhamrop B, Mootsikapun P, Boonyaprawit P: **A multicentre, randomized, double-blind, placebo-controlled trial of primary cryptococcal meningitis prophylaxis in HIV-infected patients with severe immune deficiency**. *HIV Med* 2004, **5**(3):140-143.

211. Goldman M, Cloud GA, Smedema M, LeMonte A, Connolly P, McKinsey DS, Kauffman CA, Moskovitz B, Wheat LJ: **Does long-term itraconazole prophylaxis result in in vitro azole resistance in mucosal Candida albicans isolates from persons with advanced human immunodeficiency virus infection? The National Institute of Allergy and Infectious Diseases Mycoses study group**. *Antimicrob Agents Chemother* 2000, **44**(6):1585-1587.

212. El-Sadr WM, Lundgren JD, Neaton JD, Gordin F, Abrams D, Arduino RC, Babiker A, Burman W, Clumeck N, Cohen CJ *et al*: **CD4+ count-guided interruption of antiretroviral treatment**. *N Engl J Med* 2006, **355**(22):2283-2296.

213. Chen ZM, Jiang LX, Chen YP, Xie JX, Pan HC, Peto R, Collins R, Liu LS: **Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial.** *Lancet* 2005, **366**(9497):1607-1621.

214. Chen ZM, Pan HC, Chen YP, Peto R, Collins R, Jiang LX, Xie JX, Liu LS: **Early intravenous then oral metoprolol in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial.** *Lancet* 2005, **366**(9497):1622-1632.

215. **Key Development Data and Statistics - Viet Nam** [http://web.worldbank.org/WBSITE/EXTERNAL/DATASTATISTICS/0,contentMDK:20535285~menuPK:1192694~pagePK:64133150~piPK:64133175~theSitePK:239419,00.html]

216. **Statistics at a glance: Viet Nam** [http://www.unicef.org/infobycountry/vietnam_statistics.html]

217. Hien TT, White NJ: **Qinghaosu.** *Lancet* 1993, **341**(8845):603-608.

218. Hien TT, Arnold K, Vinh H, Cuong BM, Phu NH, Chau TT, Hoa NT, Chuong LV, Mai NT, Vinh NN *et al*: **Comparison of artemisinin suppositories with intravenous artesunate and intravenous quinine in the treatment of cerebral malaria.** *Trans R Soc Trop Med Hyg* 1992, **86**(6):582-583.

219. Hien TT, Phu NH, Mai NT, Chau TT, Trang TT, Loc PP, Cuong BM, Dung NT, Vinh H, Waller DJ *et al*: **An open randomized comparison of intravenous and intramuscular artesunate in severe falciparum malaria.** *Trans R Soc Trop Med Hyg* 1992, **86**(6):584-585.

220. Trang TT, Phu NH, Vinh H, Hien TT, Cuong BM, Chau TT, Mai NT, Waller DJ, White NJ: **Acute renal failure in patients with severe falciparum malaria.** *Clin Infect Dis* 1992, **15**(5):874-880.

221. Long HT, Hibberd ML, Hien TT, Dung NM, Van Ngoc T, Farrar J, Wills B, Simmons CP: **Patterns of gene transcript abundance in the blood of children with severe or uncomplicated dengue highlight differences in disease evolution and host response to dengue virus infection.** *J Infect Dis* 2009, **199**(4):537-546.

222. Hang VT, Nguyet NM, Trung DT, Tricou V, Yoksan S, Dung NM, Van Ngoc T, Hien TT, Farrar J, Wills B *et al*: **Diagnostic Accuracy of NS1 ELISA and Lateral Flow Rapid Tests for Dengue Sensitivity, Specificity and Relationship to Viraemia and Antibody Responses.** *PLoS Negl Trop Dis* 2009, **3**(1):e360.

223. Mai NT, Hoa NT, Nga TV, Linh le D, Chau TT, Sinh DX, Phu NH, Chuong LV, Diep TS, Campbell J *et al*: ***Streptococcus suis*** **meningitis in adults in Vietnam.** *Clin Infect Dis* 2008, **46**(5):659-667.

224. Hien TT, Truong NT, Minh NH, Dat HD, Dung NT, Hue NT, Dung TK, Tuan PQ, Campbell JI, Farrar JJ *et al*: **A randomized controlled pilot study of artesunate versus triclabendazole for human fascioliasis in central Vietnam.** *Am J Trop Med Hyg* 2008, **78**(3):388-392.

225. Torok ME, Chau TT, Mai PP, Phong ND, Dung NT, Chuong LV, Lee SJ, Caws M, de Jong MD, Hien TT *et al*: **Clinical and microbiological features of HIV-**

associated tuberculous meningitis in Vietnamese adults. *PLoS One* 2008, **3**(3):e1772.

226. Caws M, Thwaites G, Dunstan S, Hawn TR, Lan NT, Thuong NT, Stepniewska K, Huyen MN, Bang ND, Loc TH *et al*: **The influence of host and bacterial genotype on the development of disseminated disease with *Mycobacterium tuberculosis***. *PLoS Pathog* 2008, **4**(3):e1000034.

227. Schultsz C, Vien le M, Campbell JI, Chau NV, Diep TS, Hoang NV, Nga TT, Savelkoul P, Stepnieuwska K, Parry C *et al*: **Changes in the nasal carriage of drug-resistant *Streptococcus pneumoniae* in urban and rural Vietnamese schoolchildren**. *Trans R Soc Trop Med Hyg* 2007, **101**(5):484-492.

228. Parry CM, Ho VA, Phuong le T, Bay PV, Lanh MN, Tung le T, Tham NT, Wain J, Hien TT, Farrar JJ: **Randomized controlled comparison of ofloxacin, azithromycin, and an ofloxacin-azithromycin combination for treatment of multidrug-resistant and nalidixic acid-resistant typhoid fever**. *Antimicrob Agents Chemother* 2007, **51**(3):819-825.

229. Flohr C, Tuyen LN, Lewis S, Minh TT, Campbell J, Britton J, Williams H, Hien TT, Farrar J, Quinnell RJ: **Low efficacy of mebendazole against hookworm in Vietnam: two randomized controlled trials**. *Am J Trop Med Hyg* 2007, **76**(4):732-736.

230. Thuong NT, Hawn TR, Thwaites GE, Chau TT, Lan NT, Quy HT, Hieu NT, Aderem A, Hien TT, Farrar JJ *et al*: **A polymorphism in human TLR2 is associated with increased susceptibility to tuberculous meningitis**. *Genes Immun* 2007, **8**(5):422-428.

231. Simmons CP, Chau TN, Thuy TT, Tuan NM, Hoang DM, Thien NT, Lien le B, Quy NT, Hieu NT, Hien TT *et al*: **Maternal antibody and viral factors in the pathogenesis of dengue virus in infants**. *J Infect Dis* 2007, **196**(3):416-424.

232. Simmons CP, Thwaites GE, Quyen NT, Torok E, Hoang DM, Chau TT, Mai PP, Lan NT, Dung NH, Quy HT *et al*: **Pretreatment intracerebral and peripheral blood immune responses in Vietnamese adults with tuberculous meningitis: diagnostic value and relationship to disease severity and outcome**. *J Immunol* 2006, **176**(3):2007-2014.

233. Schultsz C, Dong VC, Chau NV, Le NT, Lim W, Thanh TT, Dolecek C, de Jong MD, Hien TT, Farrar J: **Avian influenza H5N1 and healthcare workers**. *Emerg Infect Dis* 2005, **11**(7):1158-1159.

234. Freeman HR, Mai NT, Diep TS, Parry C, Hien TT, Farrar JJ: **The role of the polymerase chain reaction in the diagnosis of bacterial meningitis in Vietnam**. *Ann Trop Med Parasitol* 2004, **98**(1):65-70.

235. Parry CM, Wuthiekanun V, Hoa NT, Diep TS, Thao LT, Loc PV, Wills BA, Wain J, Hien TT, White NJ *et al*: **Melioidosis in Southern Vietnam: clinical surveillance and environmental sampling**. *Clin Infect Dis* 1999, **29**(5):1323-1326.

236. Dung NM, Day NP, Tam DT, Loan HT, Chau HT, Minh LN, Diet TV, Bethell DB, Kneen R, Hien TT *et al*: **Fluid replacement in dengue shock syndrome: a randomized, double-blind comparison of four intravenous-fluid regimens**. *Clin Infect Dis* 1999, **29**(4):787-794.

237. Solomon T, Thao LT, Dung NM, Kneen R, Hung NT, Nisalak A, Vaughn DW, Farrar J, Hien TT, White NJ *et al*: **Rapid diagnosis of Japanese encephalitis by using an immunoglobulin M dot enzyme immunoassay**. *J Clin Microbiol* 1998, **36**(7):2030-2034.

238. Hoa NT, Diep TS, Wain J, Parry CM, Hien TT, Smith MD, Walsh AL, White NJ: **Community-acquired septicaemia in southern Viet Nam: the importance of multidrug-resistant Salmonella typhi**. *Trans R Soc Trop Med Hyg* 1998, **92**(5):503-508.

239. White NJ, Dung NM, Vinh H, Bethell D, Hien TT: **Fluoroquinolone antibiotics in children with multidrug resistant typhoid**. *Lancet* 1996, **348**(9026):547.

240. Smith MD, Duong NM, Hoa NT, Wain J, Ha HD, Diep TS, Day NP, Hien TT, White NJ: **Comparison of ofloxacin and ceftriaxone for short-course treatment of enteric fever**. *Antimicrob Agents Chemother* 1994, **38**(8):1716-1720.

241. Yen LM, Dao LM, Day NP, Waller DJ, Bethell DB, Son LH, Hien TT, White NJ: **Role of quinine in the high mortality of intramuscular injection tetanus**. *Lancet* 1994, **344**(8925):786-787.

242. VietNam TSRo: **The third country report on the following up of the implementation to the declaration of commitment on HIV and AIDS.** In. Edited by Ministry of Health VN: Ministry of Health Viet Nam; 2008.

243. **HIV in Viet Nam - Facts and Figures** [http://www.unaids.org.vn/sitee/index.php?option=com_content&task=blogcategory&id=13&Itemid=27]

244. Al-Nasiry S, Geusens N, Hanssens M, Luyten C, Pijnenborg R: **The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells**. *Hum Reprod* 2007, **22**(5):1304-1309.

245. Wen H, R. Caldarelli-Stefano, A. M. Tortorano, P. Ferrante, and M. A., Viviani.: **A simplified method to extract high-quality DNA from *Cryptococcus neoformans***. *J Mycol Med* 1996, **6**:136-138.

246. Halliday CL, Carter DA: **Clonal reproduction and limited dispersal in an environmental population of *Cryptococcus neoformans* var *gattii* isolates from Australia**. *JClinMicrobiol* 2003, **41**(2):703-711.

247. **R: A Language and Environment for Statistical Computing (version 2.9.0). Available: http://www.R-project.org/>. Accessed 10 October 2009.** [www.R-project.org]

248. Perfect JR, Wong B, Chang YC, Kwon-Chung KJ, Williamson PR: ***Cryptococcus neoformans*: virulence and host defences**. *MedMycol* 1998, **36 Suppl 1**:79-86.

249. Higgins JP, Thompson SG: **Quantifying heterogeneity in a meta-analysis**. *Stat Med* 2002, **21**(11):1539-1558.

250. Higgins JP, Thompson SG, Deeks JJ, Altman DG: **Measuring inconsistency in meta-analyses**. *Bmj* 2003, **327**(7414):557-560.

251. Altman DG, Royston P: **The cost of dichotomising continuous variables**. *BMJ* 2006, **332**(7549):1080.

252. Royston P, Altman DG, Sauerbrei W: **Dichotomizing continuous predictors in multiple regression: a bad idea**. *Stat Med* 2006, **25**(1):127-141.

253. Austin P, Tu J: **Bootstrap methods for developing predictive models.** *The American Statistician* 2004, **58**:131 - 137.

254. Larsen RA, Bozzette SA, Jones BE, Haghighat D, Leal MA, Forthal D, Bauer M, Tilles JG, McCutchan JA, Leedom JM: **Fluconazole combined with flucytosine for treatment of cryptococcal meningitis in patients with AIDS.** *ClinInfectDis* 1994, **19**(4):741-745.

255. Leenders AC, Reiss P, Portegies P, Clezy K, Hop WC, Hoy J, Borleffs JC, Allworth T, Kauffmann RH, Jones P *et al*: **Liposomal amphotericin B (AmBisome) compared with amphotericin B both followed by oral fluconazole in the treatment of AIDS-associated cryptococcal meningitis.** *AIDS* 1997, **11**(12):1463-1471.

256. Shih CC, Chen YC, Chang SC, Luh KT, Hsieh WC: **Cryptococcal meningitis in non-HIV-infected patients.** *QJM* 2000, **93**(4):245-251.

257. Moosa MY, Coovadia YM: **Cryptococcal meningitis in Durban, South Africa: a comparison of clinical features, laboratory findings, and outcome for human immunodeficiency virus (HIV)-positive and HIV-negative patients.** *ClinInfectDis* 1997, **24**(2):131-134.

258. Mwaba P, Mwansa J, Chintu C, Pobee J, Scarborough M, Portsmouth S, Zumla A: **Clinical presentation, natural history, and cumulative death rates of 230 adults with primary cryptococcal meningitis in Zambian AIDS patients treated under local conditions.** *Postgrad Med J* 2001, 77(914):769-773.

259. Rozenbaum R, Goncalves AJ: **Clinical epidemiological study of 171 cases of cryptococcosis.** *ClinInfectDis* 1994, **18**(3):369-380.

260. Darras-Joly C, Chevret S, Wolff M, Matheron S, Longuet P, Casalino E, Joly V, Chochillon C, Bedos JP: ***Cryptococcus neoformans*** **infection in France: epidemiologic features of and early prognostic parameters for 76 patients who were infected with human immunodeficiency virus.** *Clin Infect Dis* 1996, **23**(2):369-376.

261. Huynh TX, Nguyen HC, Vo MQ, Odermatt-Biays S, Degremont A, Malvy D: **[Cryptococosis and HIV/AIDS: a review of 21 cases reported in the Tropical Diseases Centre, Ho Chi Minh City (Vietnam)].** *Sante* 2003, **13**(4):225-229.

262. Kiertiburanakul S, Wirojtananugoon S, Pracharktam R, Sungkanuparph S: **Cryptococcosis in human immunodeficiency virus-negative patients.** *Int J Infect Dis* 2006, **10**(1):72-78.

263. Cochran W: **The combination of estimates from different experiments.** *Biometrics* 1954, **10**:101-109.

264. Diamond RD, Bennett JE: **Prognostic factors in cryptococcal meningitis. A study in 111 cases.** *AnnInternMed* 1974, **80**(2):176-181.

265. White M, Cirrincione C, Blevins A, Armstrong D: **Cryptococcal meningitis: outcome in patients with AIDS and patients with neoplastic disease.** *J Infect Dis* 1992, **165**(5):960-963.

266. Khanna N, Chandramuki A, Desai A, Ravi V: **Cryptococcal infections of the central nervous system: an analysis of predisposing factors, laboratory findings and outcome in patients from South India with special reference to HIV infection.** *J Med Microbiol* 1996, **45**(5):376-379.

267. Cecchini D, Ambrosioni J, Brezzo C, Corti M, Rybko A, Perez M, Poggi S, Ambroggi M: **Tuberculous meningitis in HIV-infected and non-infected patients: comparison of cerebrospinal fluid findings.** *Int J Tuberc Lung Dis* 2009, **13**(2):269-271.

268. Bland JM, Altman DG: **Multiple significance tests: the Bonferroni method.** *Bmj* 1995, **310**(6973):170.

269. Fisher D, Burrow J, Lo D, Currie B: ***Cryptococcus neoformans* in tropical northern Australia: predominantly variant *gattii* with good outcomes.** *Aust N Z J Med* 1993, **23**(6):678-682.

270. Dromer F, Mathoulin S, Dupont B, Laporte A: **Epidemiology of cryptococcosis in France: a 9-year survey (1985-1993). French Cryptococcosis Study Group.** *ClinInfectDis* 1996, **23**(1):82-90.

271. Kwon-Chung KJ, Bennett JE: **Epidemiologic differences between the two varieties of *Cryptococcus neoformans*.** *AmJEpidemiol* 1984, **120**(1):123-130.

272. Henderson DK, Edwards JE, Dismukes WE, Bennett JE: **Meningitis produced by different serotypes of *Cryptococcus neoformans*.** In: *81st Annual Meeting of the American Society of Microbiology.* Washington D.C.; 1981.

273. Chen J, Varma A, Diaz MR, Litvintseva AP, Wollenberg KK, Kwon-Chung KJ: ***Cryptococcus neoformans* strains and infection in apparently immunocompetent patients, China.** *Emerg Infect Dis* 2008, **14**(5):755-762.

274. Longley N, Muzoora C, Taseera K, Mwesigye J, Rwembembera J, Chakera A, Wall E, Andia I, Jaffar S, Harrison T, S.: **Dose response effect of high-dose**

fluconazole for HIV-associated cryptococcal meningitis in southwestern Uganda. *Clin Infect Dis* 2008, **47**:1556-1561.

275. Stevens DA, Denning DW, Shatsky S, Armstrong RW, Adler JD, Lewis BH: **Cryptococcal meningitis in the immunocompromised host: intracranial hypertension and other complications.** *Mycopathologia* 1999, **146**(1):1-8.

276. Drutz DJ, Huppert M, Sun SH, McGuire WL: **Human sex hormones stimulate the growth and maturation of Coccidioides immitis.** *Infect Immun* 1981, **32**(2):897-907.

277. Powell BL, Drutz DJ, Huppert M, Sun SH: **Relationship of progesterone- and estradiol-binding proteins in *Coccidioides immitis* to coccidioidal dissemination in pregnancy.** *Infect Immun* 1983, **40**(2):478-485.

278. Altman DG: **Practical Statistics for Medical Research**, 1st edn. London: Chapman & Hall; 1999.

279. Harrell Jr FE: **Regression Modelling Strategies**: Springer; 2001.

280. Savelkoul PH, Aarts HJ, de Haas J, Dijkshoorn L, Duim B, Otsen M, Rademaker JL, Schouls L, Lenstra JA: **Amplified-fragment length polymorphism analysis: the state of an art.** *JClinMicrobiol* 1999, **37**(10):3083-3091.

281. Arnold C, Metherell L, Willshaw G, Maggs A, Stanley J: **Predictive fluorescent amplified-fragment length polymorphism analysis of *Escherichia coli*: high-resolution typing method with phylogenetic significance.** *JClinMicrobiol* 1999, **37**(5):1274-1279.

282. Franzot SP, Hamdan JS, Currie BP, Casadevall A: **Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for both**

local genetic differences and a global clonal population structure. *JClinMicrobiol* 1997, **35**(9):2243-2251.

283. Franzot SP, Fries BC, Cleare W, Casadevall A: **Genetic relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D.** *JClinMicrobiol* 1998, **36**(8):2200-2204.

284. Stephen C, Lester S, Black W, Fyfe M, Raverty S: **Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia.** *Can Vet J* 2002, **43**(10):792-794.

285. Upton A, Fraser JA, Kidd SE, Bretz C, Bartlett KH, Heitman J, Marr KA: **First contemporary case of human infection with *Cryptococcus gattii* in Puget Sound: evidence for spread of the Vancouver Island outbreak.** *J Clin Microbiol* 2007, **45**(9):3086-3088.

286. Chen SC, Currie BJ, Campbell HM, Fisher DA, Pfeiffer TJ, Ellis DH, Sorrell TC: ***Cryptococcus neoformans* var. *gattii* infection in northern Australia: existence of an environmental source other than known host eucalypts.** *Trans R Soc Trop Med Hyg* 1997, **91**(5):547-550.

287. Lalloo D, Fisher D, Naraqi S, Laurenson I, Temu P, Sinha A, Saweri A, Mavo B: **Cryptococcal meningitis (*C. neoformans* var. *gattii*) leading to blindness in previously healthy Melanesian adults in Papua New Guinea.** *Q J Med* 1994, **87**(6):343-349.

288. Millward IR, Williams MC: ***Cryptococcus neoformans* granuloma in the lung and spinal cord of a free-ranging cheetah (*Acinonyx jubatus*). A clinical report and literature review.** *J S Afr Vet Assoc* 2005, **76**(4):228-232.

289. Fox DL, Muller NL: **Pulmonary cryptococcosis in immunocompetent patients: CT findings in 12 patients.** *AJR Am J Roentgenol* 2005, **185**(3):622-626.

290. Torda A, Kumar RK, Jones PD: **The pathology of human and murine pulmonary infection with *Cryptococcus neoformans* var. *gattii*.** *Pathology* 2001, **33**(4):475-478.

291. Mitchell DH, Sorrell TC, Allworth AM, Heath CH, McGregor AR, Papanaoum K, Richards MJ, Gottlieb T: **Cryptococcal disease of the CNS in immunocompetent hosts: influence of cryptococcal variety on clinical manifestations and outcome.** *Clin Infect Dis* 1995, **20**(3):611-616.

292. Mitchell DH, Sorrell TC: **Pancoast's syndrome due to pulmonary infection with *Cryptococcus neoformans* variety *gattii*.** *Clin Infect Dis* 1992, **14**(5):1142-1144.

293. Lehmann PF, Morgan RJ, Freimer EH: **Infection with *Cryptococcus neoformans* var. *gattii* leading to a pulmonary cryptococcoma and meningitis.** *J Infect* 1984, **9**(3):301-306.

294. Polacheck I, Kwon-Chung KJ: **Canavanine resistance in *Cryptococcus neoformans*.** *Antimicrob Agents Chemother* 1986, **29**(3):468-473.

295. Khan ZU, Al Anezi AA, Chandy R, Xu J: **Disseminated cryptococcosis in an AIDS patient caused by a canavanine- resistant strain of *Cryptococcus neoformans* var. *grubii*.** *JMedMicrobiol* 2003, **52**(Pt 3):271-275.

296. Jain N, Wickes BL, Keller SM, Fu J, Casadevall A, Jain P, Ragan MA, Banerjee U, Fries BC: **Molecular epidemiology of clinical *Cryptococcus neoformans* strains from India**. *J Clin Microbiol* 2005, **43**(11):5733-5742.

297. Bertout S, Renaud F, Swinne D, Mallie M, Bastide JM: **Genetic multilocus studies of different strains of *Cryptococcus neoformans*: taxonomy and genetic structure**. *JClinMicrobiol* 1999, **37**(3):715-720.

298. Boekhout T, van Belkum A, Leenders AC, Verbrugh HA, Mukamurangwa P, Swinne D, Scheffers WA: **Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects**. *IntJSystBacteriol* 1997, **47**(2):432-442.

299. Chen S, Sorrell T, Nimmo G, Speed B, Currie B, Ellis D, Marriott D, Pfeiffer T, Parr D, Byth K: **Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus neoformans* in Australia and New Zealand. Australasian Cryptococcal Study Group**. *Clin Infect Dis* 2000, **31**(2):499-508.

300. Dromer F, Varma A, Ronin O, Mathoulin S, Dupont B: **Molecular typing of *Cryptococcus neoformans* serotype D clinical isolates**. *JClinMicrobiol* 1994, **32**(10):2364-2371.

301. Feng X, Yao Z, Ren D, Liao W, Wu J: **Genotype and mating type analysis of *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from China that mainly originated from non-HIV-infected patients**. *FEMS Yeast Res* 2008.

302. McEwen JG, Taylor JW, Carter D, Xu J, Felipe MS, Vilgalys R, Mitchell TG, Kasuga T, White T, Bui T *et al*: **Molecular typing of pathogenic fungi**. *MedMycol* 2000, **38 Suppl 1**:189-197.

303. Meyer W, Marszewska K, Amirmostofian M, Igreja RP, Hardtke C, Methling K, Viviani MA, Chindamporn A, Sukroongreung S, John MA *et al*: **Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA-a pilot study to standardize techniques on which to base a detailed epidemiological survey**. *Electrophoresis* 1999, **20**(8):1790-1799.

304. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M *et al*: **AFLP: a new technique for DNA fingerprinting**. *Nucleic Acids Res* 1995, **23**(21):4407-4414.

305. Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, Fisher M, Gilgado F, Hagen F, Kaocharoen S *et al*: **Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii***. *Med Mycol* 2009:1-14.

306. Litvintseva AP, Mitchell TG: **Most environmental isolates of *Cryptococcus neoformans* var. *grubii* (serotype A) are not lethal for mice**. *Infect Immun* 2009, **77**(8):3188-3195.

307. Litvintseva AP, Thakur R, Vilgalys R, Mitchell TG: **Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a unique population in Botswana**. *Genetics* 2006, **172**(4):2223-2238.

308. Mueller UG, Wolfenbarger LL: **AFLP genotyping and fingerprinting.** *Trends Ecol Evol* 1999, **14**(10):389-394.

309. Page RDM, Holmes EC: **Molecular evolution: a phylogenetic approach.**: Blackwell Science; 1998.

310. Saitou N, Nei M: **The neighbor-joining method: a new method for reconstructing phylogenetic trees.** *Mol Biol Evol* 1987, **4**(4):406-425.

311. Tamura K, Nei M, Kumar S: **Prospects for inferring very large phylogenies by using the neighbor-joining method.** *Proc Natl Acad Sci U S A* 2004, **101**(30):11030-11035.

312. St. John K, Warnow T, Moret B, Vawter L: **Performance study of phylogenetic methods: (unweighted) quartet methods and neighbor joining.** *Journal of Algorithms* 2003, **48**(1):173.

313. Daniels DL, de Wet JR, Blattner FR: **New map of bacteriophage lambda DNA.** *J Virol* 1980, **33**(1):390-400.

314. Litvintseva AP, Lin X, Templeton I, Heitman J, Mitchell TG: **Many globally isolated AD hybrid strains of *Cryptococcus neoformans* originated in Africa.** *PLoS Pathog* 2007, **3**(8):e114.

315. Litvintseva AP, Kestenbaum L, Vilgalys R, Mitchell TG: **Comparative analysis of environmental and clinical populations of *Cryptococcus neoformans*.** *J Clin Microbiol* 2005, **43**(2):556-564.

316. Bii CC, Makimura K, Abe S, Taguchi H, Mugasia OM, Revathi G, Wamae NC, Kamiya S: **Antifungal drug susceptibility of *Cryptococcus neoformans* from clinical sources in Nairobi, Kenya.** *Mycoses* 2007, **50**(1):25-30.

317. Mahmoud YA: **First environmental isolation of *Cryptococcus neoformans* var. *neoformans* and var. gatti from the Gharbia Governorate, Egypt.** *Mycopathologia* 1999, **148**(2):83-86.

318. Bennett JE, Kwon-Chung KJ, Howard DH: **Epidemiologic differences among serotypes of *Cryptococcus neoformans*.** *AmJEpidemiol* 1977, **105**(6):582-586.

319. Dromer F, Mathoulin S, Dupont B, Letenneur L, Ronin O: **Individual and environmental factors associated with infection due to *Cryptococcus neoformans* serotype D. French Cryptococcosis Study Group.** *ClinInfectDis* 1996, **23**(1):91-96.

320. Laurenson IF, Lalloo DG, Naraqi S, Seaton RA, Trevett AJ, Matuka A, Kevau IH: ***Cryptococcus neoformans* in Papua New Guinea: a common pathogen but an elusive source.** *J Med Vet Mycol* 1997, **35**(6):437-440.

321. Levitz SM: **The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcosis.** *Rev Infect Dis* 1991, **13**(6):1163-1169.

322. Van Dam MG, Seaton RA, Hamilton AJ: **Analysis of HLA association in susceptibility to infection with *Cryptococcus neoformans* var. *gattii* in a Papua New Guinean population.** *Med Mycol* 1998, **36**(3):185-188.

323. Arnold C, Metherell L, Clewley JP, Stanley J: **Predictive modelling of fluorescent AFLP: a new approach to the molecular epidemiology of *E. coli*.** *ResMicrobiol* 1999, **150**(1):33-44.

324. Varma A, Swinne D, Staib F, Bennett JE, Kwon-Chung KJ: **Diversity of DNA fingerprints in *Cryptococcus neoformans*.** *J Clin Microbiol* 1995, **33**(7):1807-1814.

325. Varma A, Wu S, Guo N, Liao W, Lu G, Li A, Hu Y, Bulmer G, Kwon-Chung KJ: **Identification of a novel gene, URE2, that functionally complements a urease-negative clinical strain of *Cryptococcus neoformans***. *Microbiology* 2006, **152**(Pt 12):3723-3731.

326. Pandya GA, Holmes MH, Petersen JM, Pradhan S, Karamycheva SA, Wolcott MJ, Molins C, Jones M, Schriefer ME, Fleischmann RD *et al*: **Whole genome single nucleotide polymorphism based phylogeny of *Francisella tularensis* and its application to the development of a strain typing assay**. *BMC Microbiol* 2009, **9**:213.

327. Kingsley RA, Msefula CL, Thomson NR, Kariuki S, Holt KE, Gordon MA, Harris D, Clarke L, Whitehead S, Sangal V *et al*: **Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype**. *Genome Res* 2009, **19**(12):2279-2287.

328. Ioerger TR, Koo S, No EG, Chen X, Larsen MH, Jacobs WR, Jr., Pillay M, Sturm AW, Sacchettini JC: **Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa**. *PLoS One* 2009, **4**(11):e7778.

329. Chen SC, O'Donnell ML, Gordon S, Gilbert GL: **Antifungal susceptibility testing using the E test: comparison with the broth macrodilution technique**. *J Antimicrob Chemother* 1996, **37**(2):265-273.

330. Espinel-Ingroff A: **Comparison of the E-test with the NCCLS M38-P method for antifungal susceptibility testing of common and emerging pathogenic filamentous fungi**. *J Clin Microbiol* 2001, **39**(4):1360-1367.

331. Espinel-Ingroff A, Fothergill A, Ghannoum M, Manavathu E, Ostrosky-Zeichner L, Pfaller M, Rinaldi M, Schell W, Walsh T: **Quality control and reference guidelines for CLSI broth microdilution susceptibility method (M 38-A document) for amphotericin B, itraconazole, posaconazole, and voriconazole**. *J Clin Microbiol* 2005, **43**(10):5243-5246.

332. Espinel-Ingroff A, Kish CW, Jr., Kerkering TM, Fromtling RA, Bartizal K, Galgiani JN, Villareal K, Pfaller MA, Gerarden T, Rinaldi MG *et al*: **Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests**. *J Clin Microbiol* 1992, **30**(12):3138-3145.

333. Ghannoum MA, Ibrahim AS, Fu Y, Shafiq MC, Edwards JE, Jr., Criddle RS: **Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique**. *J Clin Microbiol* 1992, **30**(11):2881-2886.

334. Lopez-Jodra O, Torres-Rodriguez JM, Mendez-Vasquez R, Ribas-Forcadell E, Morera-Lopez Y, Baro-Tomas T, Alia-Aponte C: **In vitro susceptibility of *Cryptococcus neoformans* isolates to five antifungal drugs using a colorimetric system and the reference microbroth method**. *J Antimicrob Chemother* 2000, **45**(5):645-649.

335. Lozano-Chiu M, Lancaster MV, Rex JH: **Evaluation of a colorimetric method for detecting amphotericin B-resistant *Candida* isolates**. *Diagn Microbiol Infect Dis* 1998, **31**(3):417-424.

336. Moraes EM, Primola NS, Hamdan JS: **Antifungal susceptibility of clinical and environmental isolates of *Cryptococcus neoformans* to four antifungal drugs determined by two techniques**. *Mycoses* 2003, **46**(5-6):164-168.

337. Petrou MA, Shanson DC: **Susceptibility of *Cryptococcus neoformans* by the NCCLS microdilution and Etest methods using five defined media.** *J Antimicrob Chemother* 2000, **46**(5):815-818.

338. Pfaller MA, Arikan S, Lozano-Chiu M, Chen Y, Coffman S, Messer SA, Rennie R, Sand C, Heffner T, Rex JH *et al*: **Clinical evaluation of the ASTY colorimetric microdilution panel for antifungal susceptibility testing.** *J Clin Microbiol* 1998, **36**(9):2609-2612.

339. Pfaller MA, Barry AL: **Evaluation of a novel colorimetric broth microdilution method for antifungal susceptibility testing of yeast isolates.** *J Clin Microbiol* 1994, **32**(8):1992-1996.

340. Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ: **Clinical evaluation of a dried commercially prepared microdilution panel for antifungal susceptibility testing of five antifungal agents against *Candida* spp. and *Cryptococcus neoformans*.** *Diagn Microbiol Infect Dis* 2004, **50**(2):113-117.

341. Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Diekema DJ: **Evaluation of the NCCLS M44-P disk diffusion method for determining susceptibilities of 276 clinical isolates of *Cryptococcus neoformans* to fluconazole.** *J Clin Microbiol* 2004, **42**(1):380-383.

342. Posteraro B, Romano L, Sanguinetti M, Masucci L, Morace G, Fadda G: **Commercial systems for fluconazole susceptibility testing of yeasts: comparison with the broth microdilution method.** *Diagn Microbiol Infect Dis* 2000, **38**(1):29-36.

343. Sanati H, Messer SA, Pfaller M, Witt M, Larsen R, Espinel-Ingroff A, Ghannoum M: **Multicenter evaluation of broth microdilution method for susceptibility testing of *Cryptococcus neoformans* against fluconazole**. *J Clin Microbiol* 1996, **34**(5):1280-1282.

344. Simor AE, Goswell G, Louie L, Lee M, Louie M: **Antifungal susceptibility testing of yeast isolates from blood cultures by microbroth dilution and the E test**. *Eur J Clin Microbiol Infect Dis* 1997, **16**(9):693-697.

345. Swinne D, Raes-Wuytack C, Van Looveren K, Desmet P: **Comparative evaluation of Fungitest-, Neo-Sensitabs- and M27T-NCCLS broth microdilution methods for antifungal drug susceptibility testing of *Candida* species and *Cryptococcus neoformans***. *Mycoses* 1999, **42**(4):231-237.

346. Warnock DW, Johnson EM, Rogers TR: **Multi-centre evaluation of the Etest method for antifungal drug susceptibility testing of *Candida* spp. and *Cryptococcus neoformans*. BSAC Working Party on Antifungal Chemotherapy**. *J Antimicrob Chemother* 1998, **42**(3):321-331.

347. Pfaller MA, Messer SA, Hollis RJ, Espinel-Ingroff A, Ghannoum MA, Plavan H, Killian SB, Knapp CC: **Multisite reproducibility of MIC results by the Sensititre YeastOne colorimetric antifungal susceptibility panel**. *Diagn Microbiol Infect Dis* 1998, **31**(4):543-547.

348. Jessup CJ, Pfaller MA, Messer SA, Zhang J, Tumberland M, Mbidde EK, Ghannoum MA: **Fluconazole susceptibility testing of *Cryptococcus neoformans*: comparison of two broth microdilution methods and clinical**

correlates among isolates from Ugandan AIDS patients. *J Clin Microbiol* 1998, **36**(10):2874-2876.

349. Witt MD, Lewis RJ, Larsen RA, Milefchik EN, Leal MA, Haubrich RH, Richie JA, Edwards JE, Jr., Ghannoum MA: **Identification of patients with acute AIDS-associated cryptococcal meningitis who can be effectively treated with fluconazole: the role of antifungal susceptibility testing.** *Clin Infect Dis* 1996, **22**(2):322-328.

350. Royston P, Parmar MK: **Flexible parametric proportional-hazards and proportional-odds models for censored survival data, with application to prognostic modelling and estimation of treatment effects.** *Stat Med* 2002, **21**(15):2175-2197.

351. Pinheiro J, Bates D: **Mixed Effects Models in S and S-Plus.** New York: Springer; 2002.

352. Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Doern GV, Diekema DJ: **Global trends in the antifungal susceptibility of *Cryptococcus neoformans* (1990 to 2004).** *J Clin Microbiol* 2005, **43**(5):2163-2167.

353. Espinel-Ingroff A: **Clinical utility of in vitro antifungal susceptibility testing.** *Rev Esp Quimioter* 2000, **13**(2):161-166.

354. Hospenthal DR, Murray CK, Rinaldi MG: **The role of antifungal susceptibility testing in the therapy of candidiasis.** *Diagn Microbiol Infect Dis* 2004, **48**(3):153-160.

355. Krcmery V, Jr.: **Is there in vivo-in vitro correlation between antifungal susceptibility, species of *Candida* spp. and clinical outcome?** *Int J Antimicrob Agents* 2000, **16**(4):537-539.

356. Archibald LK, Tuohy MJ, Wilson DA, Nwanyanwu O, Kazembe PN, Tansuphasawadikul S, Eampokalap B, Chaovavanich A, Reller LB, Jarvis WR *et al*: **Antifungal susceptibilities of *Cryptococcus neoformans*.** *Emerg Infect Dis* 2004, **10**(1):143-145.

357. Larsen RA, Bauer M, Brouwer AE, Sanchez A, Thomas AM, Rajanuwong A, Chierakul W, Peacock SJ, Day N, White NJ *et al*: **In vitro-clinical correlations for amphotericin B susceptibility in AIDS-associated cryptococcal meningitis.** *Antimicrob Agents Chemother* 2007, **51**(1):343-345.

358. Souza LK, Fernandes Ode F, Kobayashi CC, Passos XS, Costa CR, Lemos JA, Souza-Junior AH, Silva Mdo R: **Antifungal susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* in Goiania city, Goias, Brazil.** *Rev Inst Med Trop Sao Paulo* 2005, **47**(5):253-256.

359. Sar B, Monchy D, Vann M, Keo C, Sarthou JL, Buisson Y: **Increasing in vitro resistance to fluconazole in *Cryptococcus neoformans* Cambodian isolates: April 2000 to March 2002.** *J Antimicrob Chemother* 2004, **54**(2):563-565.

360. Maxwell MJ, Messer SA, Hollis RJ, Diekema DJ, Pfaller MA: **Evaluation of Etest method for determining voriconazole and amphotericin B MICs for 162 clinical isolates of *Cryptococcus neoformans*.** *J Clin Microbiol* 2003, **41**(1):97-99.

361. Tay ST, Tanty Haryanty T, Ng KP, Rohani MY, Hamimah H: **In vitro susceptibilities of Malaysian clinical isolates of *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii* to five antifungal drugs**. *Mycoses* 2006, **49**(4):324-330.

362. Perkins A, Gomez-Lopez A, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M: **Rates of antifungal resistance among Spanish clinical isolates of *Cryptococcus neoformans* var. *neoformans***. *J Antimicrob Chemother* 2005, **56**(6):1144-1147.

363. Hospenthal DR, Bennett JE: **Flucytosine monotherapy for cryptococcosis**. *Clin Infect Dis* 1998, **27**(2):260-264.

364. Schwarz P, Janbon G, Dromer F, Lortholary O, Dannaoui E: **Combination of amphotericin B with flucytosine is active in vitro against flucytosine-resistant isolates of *Cryptococcus neoformans***. *Antimicrob Agents Chemother* 2007, **51**(1):383-385.

365. Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ: **In vitro susceptibilities of clinical isolates of *Candida* species, *Cryptococcus neoformans*, and Aspergillus species to itraconazole: global survey of 9,359 isolates tested by clinical and laboratory standards institute broth microdilution methods**. *J Clin Microbiol* 2005, **43**(8):3807-3810.

366. Saag MS, Cloud GA, Graybill JR, Sobel JD, Tuazon CU, Johnson PC, Fessel WJ, Moskovitz BL, Wiesinger B, Cosmatos D *et al*: **A comparison of itraconazole versus fluconazole as maintenance therapy for AIDS-associated**

cryptococcal meningitis. National Institute of Allergy and Infectious Diseases Mycoses Study Group. *Clin Infect Dis* 1999, **28**(2):291-296.

367. Perfect JR, Durack DT: **Penetration of imidazoles and triazoles into cerebrospinal fluid of rabbits**. *J Antimicrob Chemother* 1985, **16**(1):81-86.

368. Franzot SP, Hamdan JS: **In vitro susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* to five antifungal drugs**. *Antimicrob Agents Chemother* 1996, **40**(3):822-824.

369. Almeida AM, Matsumoto MT, Baeza LC, de Oliveira ESRB, Kleiner AA, Melhem Mde S, Mendes Giannini MJ: **Molecular typing and antifungal susceptibility of clinical sequential isolates of *Cryptococcus neoformans* from Sao Paulo State, Brazil**. *FEMS Yeast Res* 2007, **7**(1):152-164.

370. Hof H: **Critical annotations to the use of azole antifungals for plant protection**. *Antimicrob Agents Chemother* 2001, **45**(11):2987-2990.

371. Serfling A, Wohlrab J, Deising HB: **Treatment of a clinically relevant plant-pathogenic fungus with an agricultural azole causes cross-resistance to medical azoles and potentiates caspofungin efficacy**. *Antimicrob Agents Chemother* 2007, **51**(10):3672-3676.

372. Drummond ED, Reimao JQ, Dias AL, de Siqueira AM: **[Behaviour azole fungicide and fluconazole in *Cryptococcus neoformans* clinical and environmental isolates]**. *Rev Soc Bras Med Trop* 2007, **40**(2):209-211.

373. Hung DQ, Thiemann W: **Contamination by selected chlorinated pesticides in surface waters in Hanoi, Vietnam**. *Chemosphere* 2002, **47**(4):357-367.

374. Nishina T, Kien CN, Noi NV, Ngoc HM, Kim CS, Tanaka S, Iwasaki K: **Pesticide residues in soils, sediments, and vegetables in the Red River Delta, northern Vietnam.** *Environ Monit Assess* 2009.

375. Wrigley T: **Microbial counts and pesticide concentrations in drinking water after alum flocculation of channel feed water at the household level, in Vinh Long Province, Vietnam.** *J Water Health* 2007, **5**(1):171-178.

376. Davey KG, Johnson EM, Holmes AD, Szekely A, Warnock DW: **In-vitro susceptibility of *Cryptococcus neoformans* isolates to fluconazole and itraconazole.** *J Antimicrob Chemother* 1998, **42**(2):217-220.

377. Aller AI, Claro R, Castro C, Serrano C, Colom MF, Martin-Mazuelos E: **Antifungal susceptibility of *Cryptococcus neoformans* isolates in HIV-infected patients to fluconazole, itraconazole and voriconazole in Spain: 1994-1996 and 1997-2005.** *Chemotherapy* 2007, **53**(4):300-305.

378. Fromtling RA, Abruzzo GK, Bulmer GS: ***Cryptococcus neoformans*: comparisons of in vitro antifungal susceptibilities of serotypes AD and BC.** *Mycopathologia* 1986, **94**(1):27-30.

379. Trilles L, Fernandez-Torres B, Lazera Mdos S, Wanke B, Guarro J: **In vitro antifungal susceptibility of *Cryptococcus gattii*.** *J Clin Microbiol* 2004, **42**(10):4815-4817.

380. Chen YC, Chang SC, Shih CC, Hung CC, Luhbd KT, Pan YS, Hsieh WC: **Clinical features and in vitro susceptibilities of two varieties of *Cryptococcus neoformans* in Taiwan.** *Diagn Microbiol Infect Dis* 2000, **36**(3):175-183.

381. Gomez-Lopez A, Zaragoza O, Dos Anjos Martins M, Melhem MC, Rodriguez-Tudela JL, Cuenca-Estrella M: **In vitro susceptibility of *Cryptococcus gattii* clinical isolates**. *Clin Microbiol Infect* 2008, **14**(7):727-730.

382. Poonwan N, Mikami Y, Poosuwan S, Boon-Long J, Mekha N, Kusum M, Yazawa K, Tanaka R, Nishimura K, Konyama K: **Serotyping of *Cryptococcus neoformans* strains isolated from clinical specimens in Thailand and their susceptibility to various antifungal agents**. *Eur J Epidemiol* 1997, **13**(3):335-340.

383. Pfaller MA, Messer SA, Boyken L, Tendolkar S, Hollis RJ, Diekema DJ: **Selection of a surrogate agent (fluconazole or voriconazole) for initial susceptibility testing of posaconazole against *Candida* spp.: results from a global antifungal surveillance program**. *J Clin Microbiol* 2008, **46**(2):551-559.

384. Longley N, Muzoora C, Taseera K, Mwesigye J, Rwebembera J, Chakera A, Wall E, Andia I, Jaffar S, Harrison TS: **Dose response effect of high-dose fluconazole for HIV-associated cryptococcal meningitis in southwestern Uganda**. *Clin Infect Dis* 2008, **47**(12):1556-1561.

385. Pfaller MA, Diekema DJ, Ostrosky-Zeichner L, Rex JH, Alexander BD, Andes D, Brown SD, Chaturvedi V, Ghannoum MA, Knapp CC *et al*: **Correlation of MIC with outcome for *Candida* species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints**. *J Clin Microbiol* 2008, **46**(8):2620-2629.

386. Sloan D, Dlamini S, Paul N, Dedicoat M: **Treatment of acute cryptococcal meningitis in HIV infected adults, with an emphasis on resource-limited settings**. *Cochrane Database Syst Rev* 2008(4):CD005647.

387. Chuang YM, Ho YC, Chang HT, Yu CJ, Yang PC, Hsueh PR: **Disseminated cryptococcosis in HIV-uninfected patients**. *Eur J Clin Microbiol Infect Dis* 2008, **27**(4):307-310.

388. Litvintseva AP, Marra RE, Nielsen K, Heitman J, Vilgalys R, Mitchell TG: **Evidence of sexual recombination among *Cryptococcus neoformans* serotype A isolates in sub-Saharan Africa**. *Eukaryot Cell* 2003, **2**(6):1162-1168.

389. Litvintseva AP, Thakur R, Reller LB, Mitchell TG: **Prevalence of clinical isolates of *Cryptococcus gattii* serotype C among patients with AIDS in Sub-Saharan Africa**. *J Infect Dis* 2005, **192**(5):888-892.

390. Barchiesi F, Schimizzi AM, Caselli F, Novelli A, Fallani S, Giannini D, Arzeni D, Di Cesare S, Di Francesco LF, Fortuna M *et al*: **Interactions between triazoles and amphotericin B against *Cryptococcus neoformans***. *AntimicrobAgents Chemother* 2000, **44**(9):2435-2441.

391. Lortholary O, Improvisi L, Rayhane N, Gray F, Fitting C, Cavaillon JM, Dromer F: **Cytokine profiles of AIDS patients are similar to those of mice with disseminated *Cryptococcus neoformans* infection**. *InfectImmun* 1999, **67**(12):6314-6320.

392. Lutz JE, Clemons KV, Stevens DA: **Enhancement of antifungal chemotherapy by interferon-gamma in experimental systemic cryptococcosis**. *JAntimicrobChemother* 2000, **46**(3):437-442.

393. Clancy CJ, Nguyen MH, Alandoerffer R, Cheng S, Iczkowski K, Richardson M, Graybill JR: ***Cryptococcus neoformans* var. *grubii* isolates recovered from persons with AIDS demonstrate a wide range of virulence during murine meningoencephalitis that correlates with the expression of certain virulence factors**. *Microbiology* 2006, **152**(Pt 8):2247-2255.

394. Kelly SL, Lamb DC, Kelly DE, Manning NJ, Loeffler J, Hebart H, Schumacher U, Einsele H: **Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation**. *FEBS Lett* 1997, **400**(1):80-82.

395. Joseph-Horne T, Hollomon D, Loeffler RS, Kelly SL: **Cross-resistance to polyene and azole drugs in *Cryptococcus neoformans***. *Antimicrob Agents Chemother* 1995, **39**(7):1526-1529.

396. Chapeland-Leclerc F, Bouchoux J, Goumar A, Chastin C, Villard J, Noel T: **Inactivation of the FCY2 gene encoding purine-cytosine permease promotes cross-resistance to flucytosine and fluconazole in *Candida lusitaniae***. *Antimicrob Agents Chemother* 2005, **49**(8):3101-3108.

397. Florent M, Noel T, Ruprich-Robert G, Da Silva B, Fitton-Ouhabi V, Chastin C, Papon N, Chapeland-Leclerc F: **Nonsense and missense mutations in FCY2 and FCY1 genes are responsible for flucytosine resistance and flucytosine-fluconazole cross-resistance in clinical isolates of *Candida lusitaniae***. *Antimicrob Agents Chemother* 2009, **53**(7):2982-2990.

398. Papon N, Noel T, Florent M, Gibot-Leclerc S, Jean D, Chastin C, Villard J, Chapeland-Leclerc F: **Molecular mechanism of flucytosine resistance in**

***Candida lusitaniae*: contribution of the FCY2, FCY1, and FUR1 genes to 5-fluorouracil and fluconazole cross-resistance**. *Antimicrob Agents Chemother* 2007, **51**(1):369-371.

APPENDIX: PUBLICATIONS ARISING FROM THIS THESIS

Day JN, Farrar, JJ. Neurological Infections. J R Coll Physicians Edinb 2006; 36:344–349

Day JN. The Year in Infection 2: Meningitis. Book Chapter. Ed. Wilcox M. Clinical Publishing 2005

Day JN. Cryptococcal Meningitis. Practical Neurology Oct 2004; 4(5) 274-285

De Jong MC, Day JN, Nguyen HC. HIV/AIDS - A Vietnamese Perspective. IATEC Update July 2004, 12

Day JN, Hien TT, Farrar J. Expiry-date tampering. Lancet 2004; 363:172

Day JN, Lalloo DG. Neurological syndromes and the traveller: an approach to differential diagnosis. Journal of Neurology, Neurosurgery & Psychiatry 2004; 75 Suppl 1:i2-9.

Chau TT, Mai NT, Phu NH, Nghia HD, Chuong LV, Sinh DX, Chinh NT, Sieu TP, Duong VA, Diep PT, Tuan PQ, Campbell JI, Baker S, Hien TT, Farrar JJ, Day JN. Clinical Features and Outcomes of Cryptococcal Meningitis in Immunocompetent

Patients in Viet Nam - High Prevalence of *Cryptococcus neoformans var grubii* in the Absence of Underlying Disease. *Accepted BMC Infectious Diseases*

Jeremy N Day, Thu N Hoang, Van A Duong, Chau TT Hong, Phan T Diep, James I Campbell, Tran PM Sieu, Tran T Hien, Tien Bui, David G Lalloo, Dee Carter, Stephen Baker, Jeremy J Farrar. A sub-genotype of closely related strains of *Cryptococcus neoformans* var *grubii* causes cryptococcal meningitis in HIV uninfected patients in Vietnam. *Submitted Journal of Infectious Diseases*

doanh nhân Việt Nam cần phải ra nước ngoài hoạt động. Do đó, luật pháp Việt Nam vừa phản ánh được bản chất chế độ xã hội, truyền thống dân tộc, mà còn cả những thông lệ quốc tế đã được tất cả các dân tộc, quốc gia chấp nhận như một qui tắc điều chỉnh hành vi chung cho mọi doanh nghiệp, không phân biệt chế độ xã hội. Do đó, trong tiến trình phát triển và sử dụng khu vực FIE tiếp tục hoàn thiện hệ thống pháp luật. Trong đó, cần hoàn thiện và xây dựng đồng bộ, nhất quán các văn bản quy phạm pháp luật liên quan đến đầu tư nước ngoài. Hệ thống pháp luật phải có tính hấp dẫn, thông thoáng, rõ ràng, ổn định và mang tính cạnh tranh cao so với các nước trong khu vực. Tiếp tục hoàn chỉnh hệ thống pháp luật chung về đầu tư và Luật Doanh nghiệp thống nhất để tạo môi trường kinh doanh thật sự ổn định, bình đẳng. Tiếp tục hoàn thiện Luật về kinh doanh bất động sản, Luật Cạnh tranh và Luật Chống độc quyền... Phải coi yếu tố pháp lý vừa là một nhân tố quan trọng trong việc thu hút vốn đầu tư nước ngoài, vừa là cơ sở để giữ vững quyền tự chủ về kinh tế, chính trị của đất nước. Nghiên cứu sửa đổi hệ thống các loại thuế, hướng mọi nỗ lực về chính sách thuế vào mục đích thúc đẩy đầu tư để mở rộng khu vực FIE. Trước mắt cần hoàn thiện các đạo luật sau:

Thứ nhất, hoàn thiện về Luật Lao động: mặc dù đã cho phép DNCVĐTNN trực tiếp tuyển dụng lao động, nhưng phía Việt Nam vẫn cần can thiệp mềm dẻo vào vấn đề tuyển dụng lao động của họ. Trong đó, cần khắc phục tình trạng các DNCVĐTNN phải sử dụng lao động qua các tổ